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Allison R. Kermode *Editor*

Seed Dormancy

Methods and Protocols

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Seed Dormancy

Methods and Protocols

Edited by

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Preface

This book covers analytical methods and approaches, which have led to significant advances in our understanding of seed dormancy and germination and have the potential to lead to new avenues of research. The methods detailed in this book are of value for both applied and fundamental seed research, and range from whole seed batch treatments to the analysis of tissues or even single seeds at the DNA, protein, and metabolite levels. Methods are described in detail and critical steps explained so that several of the chapters of this book can serve both as a protocol and a guideline for their adaptation and optimization to new applications or species.

The viability and vigor of seeds are the very basis for sustainable agriculture and forestry, and understanding the molecular and cellular events underlying these processes will become increasingly important to many economical sectors and for species that provide the world's food supply. Seeds are also the focus of environmental conservation efforts aimed at preserving the floral biodiversity of vanishing habitats. Understanding seed-related processes is thus of major social, environmental, and economical concern. The main foci of seed-related research are the regulation of lifecycle transitions, such as the dormancy-to-germination and germination-to-growth transitions; seed viability, quality, and vigor are central as well, and are influenced by events that take place during seed development.

Some orthodox seeds enter a state of dormancy during their development. Like their nondormant counterparts, they become quiescent during the final stages of seed development associated with desiccation and a cessation of the vascular supply from the mother plant, in which seed metabolism is strongly reduced and meristematic activity ceases. However, unlike the mature, quiescent, nondormant seed, the dormant seed fails to complete germination under favorable conditions (e.g., adequate moisture and oxygen), even though it is viable. Thus, dormancy ensures the proper temporal (and in some cases spatial) distribution of seed germination and is critical for a species' survival in its natural habitat. Dormant seeds must be exposed to species-specific environmental cues for dormancy to be terminated; these cues include moist chilling, light of a certain wavelength, or even smoke. Dormancy release also occurs in some species during seed after-ripening, i.e., air-dry storage of seeds at room temperature.

The first step toward an understanding of the processes leading to the induction, maintenance, and termination of seed dormancy is a classification of different dormancy types, as described in Chapter 3. The dormancy classification system explained in this chapter has been detailed by Baskin and Baskin and is now widely accepted and used throughout this book. Chapter 3 also deals with a major challenge in dormancy research, namely, the fact that dormancy is in laboratory practice usually assessed by the absence of germination in a seed batch within a defined period of time, while a seed batch that germinates to 100% is classified as non-dormant. This can lead to some inaccuracies because seed viability within a population may not be 100%.

A proper balance of seed dormancy is crucial for agricultural crop production: seeds must not germinate on the mother plant, a process known as preharvest sprouting, which leads to major losses in crop production due to a decline in seed quality. On the other hand,

deeper dormancy can interfere with the ability of seeds to germinate readily when sown. Huge efforts go into optimizing the level of dormancy in cereals and other cultivated crops with these challenges in mind, and understanding the various external and internal factors that influence the depth of seed dormancy is thus critical. Chapter 2 discusses this point in detail focusing on agricultural crops, as well as the challenges faced by seed banks in the storage and preservation of a large variety of seeds with differing characteristics.

Similar issues are relevant to the forest industry, as explained in Chapter 1. As examples, some of the conifer species that are particularly important to the forest industry of British Columbia, Canada are discussed, including yellow-cedar (*Callitropsis nootkatensis*), western white pine (*Pinus monticola*), and true fir (*Abies*) species. Many species-specific protocols have been developed to break seed dormancy, as the agricultural and forest industries rely upon seeds that exhibit high germinability and vigorous, synchronous growth after germination. This work is critical to the forest industry, where certain conifer species of economic value exhibit profound dormancy, which is only terminated by a prolonged protocol – sometimes up to 4–5 months in duration. For species with profound dormancy, effective termination of dormancy appears not only to promote synchronous germination of seeds, but also to promote vigorous growth after germination. An added complication for forest species is the large genetic variability, meaning that protocols must be tested on several different seedlots and clones of a given conifer species. Chapter 4 gives an overview of protocols used for conifer species exhibiting deep dormancy, including traditional protocols and alternative protocols that are shorter in duration. Although not directly addressed in this book, understanding the optimal conditions for long-term storage of conifer seeds, as well as the genetic/environmental basis for different rates of seed deterioration during long-term storage is another issue of critical importance to the forest industry. The chapters on crop species of value to the forest and agriculture industries and the greatest challenges that impact these industries underscore the importance of conducting basic seed biology research on economically relevant species, rather than excluding these because of a preference for the more readily studied established model species (e.g., *Arabidopis thaliana*).

While the agricultural industry may consider deep dormancy to be an undesirable trait, this is not necessarily the case for conifer species as it pertains to their regeneration at natural stands. Yellow-cedar is an example of a conifer species whose natural habitat is typically at high elevation. After dispersal from the tree, the seeds of this species must rely on experiencing external cues (including prolonged cold temperatures at high moisture) to time its germination such that seedling survival is optimized. It is not clear whether such a species will be able to adapt to climate change (warming) for regeneration at natural stands, especially considering the seeds' requirement of prolonged moist chilling for effective dormancy breakage.

Obtaining relevant data from seed dormancy research conducted in the laboratory is undoubtedly a challenge. This challenge becomes even greater when one is characterizing seed behavior in the natural environment. Chapter 5 deals with some of the key factors that require careful consideration when one is embarking on molecular studies of seeds in their natural environment such that the most informative interpretation of the data is fostered.

Research into the role of hormones in the control of seed dormancy and germination has long focused on two key players: abscisic acid (ABA) and gibberellins (GAs). Exciting discoveries are still being made regarding these two antagonists, for which the ultimate downstream targets responsible for their effects on germination are only very partially known. The horizon is now widening to include the effects of other hormones, such as

ethylene. The ability to profile a range of hormones and hormone metabolites simultaneously in seed and embryo tissues, and to monitor enzymes involved in hormone catabolism (Chapters 7–9) has contributed to a more broad perspective of hormone action and has also led to significant advances in our understanding of the roles of hormone flux in the control of dormancy and germination. We also know more about the localization of hormones and hormone metabolites in plant cells and tissues, and other regulatory mechanisms that involve hormone metabolites as "storage" forms of hormones (i.e., those able to "release" the free and physiologically active hormone at a later time). This book emphasizes methods important for ABA analyses, partly because of the role of this hormone as a key regulator of dormancy and germination; this includes methods that have been important for receptor identification (Chapter 6), analyses of ABA-catabolizing enzymes (the 8′-hydroxylases) (Chapters 8 and 9), and identification of novel signal transduction components, interacting partners and/or response factors (e.g., Chapter 12). Some of the approaches can serve as a general guideline to the analysis of other hormones involved in the control of seed dormancy and germination.

Plant hormones can interact with or cause changes in the concentration of reactive oxygen species and nitric oxide. In addition to the traditional view of reactive oxygen species as damaging compounds, reactive oxygen and nitrogen species can play roles as signaling molecules or as components of signal transduction pathways (Chapter 22). For example, reactive oxygen species produced in select cells of the micropylar seed tissues may signal these cells to undergo programed cell death and/or weaken cell walls, thus promoting radicle protrusion. On another level, the redox state of seeds has also been correlated with their longevity and storage characteristics, and protein oxidation caused by reactive oxygen species during storage has been shown to play a role in seed deterioration. Methodological approaches to these issues are described in Chapters 20–22.

Epigenetic changes occur during stress responses and lifecycle transitions in plants, including the dormancy to germination transition, and this represents a new and exciting area. It has been known for some time that the conditions under which the mother plant sets seeds influence the dormancy and germination characteristics as well as the seedling performance of its offspring. These processes, including epigenetic control of gene expression, and relative dominance of maternal versus paternal genetic contributions due to selective gene silencing, are in some instances critical to seed development and viability, seed dormancy/germination, and seedling growth and stress resilience. Thus, epigenetic studies are an integral part of seed biology, and seeds of angiosperms, with their maternal seed coat, triploid endosperm, and diploid embryo present a unique system to study epigenetic and imprinting processes. The evolutionary aspects of these processes are also of great interest. MicroRNAs (Chapter 13) are involved in the attenuation of hormone signaling, and they also underlie some epigenetic processes.

Methods have been developed to analyze some key mechanistic features of epigenetic processes, including DNA methylation and histone modifications, the latter representing a highly complex pattern of posttranslational modifications. The tools for analyses of some of these modifications are covered in a comprehensive manner by Chapter 16, which also describes a novel NMR-based approach for the analysis of histone modifications. This allows the identification of a range of the modifications present on a particular type of histone. Chapter 15 focuses on methods used to analyze specific modifications to histones and the associated DNA, as well as DNA methylation. The results of these types of analyses, particularly as they pertain to lifecycle transitions and stress resilience of seedlings,

along with the identification of the genes/enzymes that mediate the epigenetic changes, are awaited with interest.

The genetic basis of seed dormancy has been investigated largely in the model species *Arabidopsis thaliana*, and Chapter 11 focuses on the methodological aspects of these genetic analyses (identification of quantitative trait loci) while Chapter 14 focuses on proteomics approaches. DNA and RNA isolation is no easy task in seeds because of their high concentrations of phenolic substances and other inhibitors; the relevant extraction procedures are described in Chapter 10.

As noted earlier, the attention of seed biologists on non-model plant species is an encouraging trend that is widening the range of seed anatomies, dormancy types, and germination control mechanisms that are being investigated. Progress in genome sequencing techniques has strongly contributed to this development, and the methods available will be useful tools for the study of other species that are not established models.

Seed dormancy has parallels to other dormancy processes in plants, such as bud dormancy. In photoperiodic trees exposed to short days in autumn, internode elongation ceases and the apex is transformed to a bud, which acquires desiccation and cold tolerance and enters a state of dormancy. Bud formation is controlled by ABA, and genes that are differentially regulated during *Arabidopsis* seed dormancy induction and apical bud development in the angiosperm model, poplar, show many parallels, suggesting similar mechanisms.

The current trends in seed biology include a heightened emphasis on the analysis of processes beyond the "level" of the whole seed, i.e., at the tissue, cellular, or even subcellular levels. This has become possible with refinements allowing for a "down-scaling" of longer standing methods as well as the development of new technologies, a selection of which are included in Chapters 17–19. This includes spectroscopic methods (some of which allow for nondestructive sampling) as well as highly effective tissue-printing methods for seed dormancy research. Imaging techniques, such as MRI and NMR, have seen huge advances during the last few years, and their use on seeds has led to important discoveries related to seed structure, water uptake and distribution (including the route of water uptake during seed imbibition), seed oil content, and selected secondary metabolites, and the relation of these parameters to seed dormancy, germination, stress resilience/vigor, and storability (resistance to deterioration). The use of these imaging techniques to elucidate additional aspects of seed biology is part of the larger trend toward using techniques traditionally associated with biophysics or engineering in biology research.

Mining the huge amount of transcriptomic and proteomic data that has been made publicly available is still a challenge, and the development of easily accessible interfaces has greatly helped those that are not adept in bioinformatics to make use of this valuable resource. A seed-specific database for the eFP-browser and a seed-specific gene ontology system are explained in Chapter 23. In connection with these bioinformatic efforts, a very recent approach to seed biology is centered on interdisciplinary collaborations with mathematicians, statisticians, and computer scientists: modeling of dormancy and germination at the whole seed, tissue or molecular levels will reveal as yet overlooked connections and insights into these highly complex processes.

The last 10 years have led to significant advances in the realm of seed dormancy and germination research and research on various other aspects of seed biology that control the viability and vigor of the next generation. All seed biologists should look forward to the next 10 years of advancement with great excitement.

Kerstin Muller and Allison R. Kermode

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Part I

Introduction

Chapter 1

Challenges Facing the Forest Industry in Relation to Seed Dormancy and Seed Quality

Michael U. Stoehr and Yousry A. El-Kassaby

Abstract

Artificial regeneration of forests through planting requires high quantities of quality seeds for growing vigorous seedlings. These seedlings are raised in nurseries, where germination capacity (GC) and speed are the most important germination parameters. Germination performance is enhanced by prescribing species-specific dormancy-breaking treatments to individual seedlots in bare-root and container nurseries. For most conifer species in British Columbia, the dormancy-breaking treatments and germination conditions have been worked out, but fine-tuning and optimization could improve germination capacity and speed of germination. Implications of inter- and intra-species variations in germination behaviour and seed quality and their influence on the development of unintentional directional selection of specific genotypes are discussed. The potential of molecular and genomics approaches to understand the underlying biology of seed germination-related problems is also discussed.

Key words: Nursery, Stratification, Germination capacity, Germination speed, Climate change, Dormancy

1. Introduction

Seeds of any plant species are produced to ensure the establishment and survival of future generations. In forestry, seeds are needed for natural regeneration after natural disturbances or harvesting, and seedlings are grown in nurseries for artificial regeneration and afforestation. Seed genetic quality and physiological attributes, such as vigour, viability, and germination behaviour, are important for artificial regeneration through the production of healthy seedlings. Seedling production failure is often associated with economic losses. Therefore, it is desirable to maximize seed utilization efficiency by converting every seed sown to a seedling.

The literature is vast in the area of general seed biology, and several books are available with detailed information on basic

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aspects of seed physiology and the biology of seed dormancy and germination (1–5). We also refer the reader to several other books that are more specific to tree seeds and their ecology (6–8).

In this chapter, we outline the implications of seed biology attributes, such as germination behaviour and dormancy, in particular, on seedling production in the forest nursery industry and highlight the challenges caused by these biological constraints. In British Columbia (BC), Canada, the nursery industry produces, on average, over 200 million seedlings yearly for reforestation programmes on Crown and private forest land (9). The BC regeneration programme has a large economic impact, considering the intensive cascading activities that must follow seedling production, such as planting, tending, and the maintenance of a competition-free environment until the legislated "free-to-grow" state is reached and seedlings attain the required 3-m height. On average, roughly 50% of the raised seedlings originate from natural stand seed collections while the remaining are grown from orchard seed with various levels of genetic gain, depending on the species and breeding zone (10).

Most of the trees grown in British Columbia for reforestation are conifers. Therefore, we emphasize the seed characteristics of conifers. We describe the different types of seed dormancy and how these affect nursery operations, highlighting the evolutionary mechanisms leading to seed dormancy. Further, seed quality, as represented by the proportion of seeds that germinate after dormancy-breaking treatments, has a direct impact on maximum germination capacity (GC). Also important is the ability of the germinated seeds to undergo vigorous early post-germinative growth leading to emergence. Germination and early growth capacity are probably the most important characteristics of a seedlot for nursery operations, and they have a strong economic impact on the profit margin of a nursery; however, germination promptness (known as lag time) is also important since it is associated with crop uniformity and the amount of energy used to maintain a favourable germination environment. As most conifers are very heterogeneous and often show high levels of genetic variation in most seed traits, dormancy and germination are no exception (6). The observed genetic variation in seed attributes of conifers, in turn, places even more complications on nurseries in order to account for and accommodate these differences in seed germination behaviour. We describe the magnitude of these effects and illustrate how the nursery industry copes with these added challenges. At the conclusion of the chapter, we outline how molecular biology and its present and emerging tools can be used to address some of the problems that the forest nursery industry is facing in light of challenges associated with seed dormancy and seed quality.

2. Seed Pretreatments

In some provinces in Canada, seed extraction, processing, sanitation treatment, testing, storage, and even dormancy-breaking treatments are carried out by provincial seed centres while in others, this responsibility falls to licensees responsible for reforestation or individual tree nurseries (B. Wang, pers. comm.). To prevent seed or seedling losses, damaging seed-borne pathogens must be eliminated. These pretreatments represent a sound preventative measure and are particularly important prior to exposing the seeds to a warm and moist environment. Kolotelo et al. (11) review the current knowledge and practices of seed sanitation.

Of equal importance to seed sanitation is the application of proper dormancy-breaking treatments, which are effective for multiple seedlots. Again, in many cases, this falls into the responsibility of individual nurseries and special care must be taken to ensure that these treatments are followed as prescribed (see below). A fact requiring special attention is that seeds that are ready to be germinated may be more susceptible to damage during handling or storage, and inadvertent desiccation of seeds prior to sowing must be avoided. Using sound sanitation management and appropriate dormancy-breaking treatments ensures that seeds reach their potential germination capacity given a favourable germination environment.

3. Seed Dormancy

Dormancy is the inability of a viable and healthy seed to germinate under otherwise suitable conditions. These conditions include sufficient water and oxygen, and suitable temperature and light quality or intensity. Generally, there are two types of dormancy recognized: endogenous and exogenous (5). These have also been referred to as embryo dormancy and coat-enhanced dormancy, respectively (4). In endogenous dormancy, conditions within the embryo prevent germination; in exogenous dormancy, structures "outside" of the embryo, such as the megagametophyte or seed coat, prevent the emergence of the radicle, in some cases, by imposing mechanical restraint. There are many classes and subclasses of dormancy (for complete coverage, see Baskin and Baskin (5)), but for the purpose of this chapter, the details of dormancy and how it may be overcome are relevant to many if not most conifers of the temperate and boreal zones. Seeds of some conifer species, such as the true firs (*Abies* spp.), yellow-cedar, and western white pine, are deeply dormant at maturity. For yellow-cedar (*Callitropsis nootkatensis*

(D. Don) Oerst.) (formerly known as *Chamaecyparis nootkatensis* ([D.Don] Spach)), the megagametophyte is the major seed tissue that imposes dormancy (12). For seeds of western white pine (*P. monticola* Dougl. ex D. Don.), both the testa and the megagametophyte contribute to the inhibition of germination of mature dormant seeds (13, 14). Nonetheless, factors within the embryo appear to contribute to some extent in seeds of both conifer species. Regardless of the general category of seed dormancy, there are several mechanistic factors that impose and maintain dormancy of conifer seeds. For yellow-cedar, mechanical restraint of the megagametophyte and the biosynthesis/action of inhibitors (particularly ABA) in the embryo and seed tissues appear to be the predominant factors. In seeds of western white pine, ABA also plays a pivotal role. Here, a change in ABA flux, i.e. in the relative capacity of seeds to affect ABA biosynthesis versus ABA catabolism, is a key controller of the dormancy-to-germination transition.

Conifers that require dormancy-breaking treatment refinements to fine-tune and optimize germination behaviour include the deeply dormant true firs (*Abies* spp.) and whitebark pine (*Pinus albicaulis* Engelm.) (Kolotelo, pers. comm.).

4. Dormancy-Breaking Treatments

There is an adaptive significance of seed dormancy in tree species that must survive winter and cold temperatures. This is related to ensuring that a seed does not germinate unless an extended period of favourable growth conditions are met (15). Most seeds of northern hemisphere conifers mature in the early fall and if germination occurs soon thereafter, this would be potentially fatal for the newly sprouted seedling. To avoid this survival bottleneck, an evolutionary safety mechanism, such as the need for a prolonged period of moist chilling (to mimic cold, wet conditions), has evolved. However, it is paramount that following dormancy-breaking treatments, favourable conditions for germination must be met so that germination occurs successfully and is synchronous within the population. Applying a uniform dormancy-breaking treatment across a seedlot enhances the chances for higher and prompt germination.

Recommended times of the moist-chilling or "stratification" treatments (from the original placement of seeds between layers or strata of moist materials, such as cloth, sand, or peat (11)) vary in length (ranging from 0 to 150 days) for most northern conifer species. Generally, imbibed seeds are placed at temperatures around 5°C for the prescribed period for each species. However, within-species variation is evident in germination behaviour due to genetic variation in levels of dormancy and germination parameters (16–18) (see also Farmer (6) for a more complete review). Genetic variation

Table 1
Moist chilling requirements for seeds of British Columbia trees and other boreal, north-temperate trees

Species	Soak period (h)	Moist chilling (d)	Germination temperature[a] (°C)	Additional comments	References
Pseudotsuga menziesii	24	21	30–20		(11)
Larix occidentalis	24	21	30–20		(11)
L. laricina		30–60	30–20		(5)
Pinus contorta, *P. ponderosa*	24	28	30–20		(11)
P. monticola	336	98	30–20		(5)
P. albicaulis		90–120	30–20		
P. banksiana		0–7	30–20		(5)
Tsuga heterophylla, *T. mertensiana*	24	28	20–20		(11)
Abies grandis	48	28	30–20		(11)
A. amabilis, *A. grandis*, *A. lasciocarpa*	48	56	30–20		(11)
A. balsamea		28	30–20		(5)
Chamaecyparis nootkatensis	48	84	30–20	Footnote[b]	(11)
Thuja plicata	0	0	30–20		(11)
Picea spp. (*P. glauca and glauca × engelmannii hybrids*, *P. sitchensis*)	24	0	30–20		(11)
P. mariana		24	20–10		(5)
Betula papyrifera		14	25–25		(5)
Alnus spp.		0	30–20		(5)
Populus spp.		0	30–20 or 5–25		(5)

[a] Eight hours at the higher temperature and 16 h at the lower temperature
[b] Twenty eight days at 20°C followed by 56 days at 2–5°C. *Chamaecyparis nootkatensis* is now known as *Callitropsis nootkatensis*

in germination parameters and dormancy places an added level of complexity causing non-uniform germination patterns in the nursery that can lead to unintentional selection of certain families especially at the time of thinning seedlings in containerized nursery operations (19, 20). Moist-chilling conditions for British Columbia's conifers are listed in Kolotelo et al. (11) and for other boreal and north-temperate tree species in Baskin and Baskin (5) and are summarized in Table 1.

In natural regeneration methods, where successful seedling establishment relies on the natural seed *rain*, dormancy is the safety mechanism that prevents "premature" germination of seedlings in the fall. The moist-chilling periods required to break dormancy are such that seeds germinate only when the conditions (usually determined by temperature, and likely also photoperiod) are favourable during the following spring. This enhances the chances for successful seedling emergence and survival; however, unpredictable environmental contingencies often occur causing reduced recruitment or in some cases total recruitment failure. Hence, artificial regeneration is often favoured.

5. Germination in the Nursery

5.1. Containerized Nursery

Germination conditions and dormancy-breaking treatments are usually explored and tested in the laboratory under closely controlled conditions with respect to temperature, light levels, and other necessary requirements, depending on the specific requirements of the species. Recently, El-Kassaby and co-workers (21) demonstrated the utility of the non-linear four-parameter Hill function (4-PHF) to estimate and biologically interpret the four parameters with respect to germination. After fitting germination data (cumulative germination percent over time), the lag or onset of germination, time of maximum germination rate, instantaneous rate of germination, and final germination percentage (germination capacity) can easily be calculated given the four parameters describing the shape of the curve of the Hill function. The ability to express the course of germination mathematically allowed the application of the integration approach of Richter and Switzer (22) to quantitatively estimate the degree of dormancy (see El-Kassaby et al. (21)). In this approach, the level of dormancy was quantified as the area between a seedlot's germination curves under stratification (moist chilling) and control (unstratified) conditions (Fig. 1). With this information, the extent of dormancy and the efficacy of dormancy-breaking treatments, as well as differences among test entries, such as provenances, families, or orchard clones, can be assessed. Other non-linear functions also allow the estimation of some of these characteristics (23–25). However, it is prudent to point out that often laboratory germination tests do not reflect greenhouse results (5) and testing under "unfavourable" conditions has been advocated to reflect the true potential of treated seed (26).

Appropriate and species-specific dormancy-breaking treatments lead to greater uniformity and greater speed of germination (see also Chapter 4). According to Kolotelo (pers. comm.), the greatest cost in greenhouse seedling production is heating

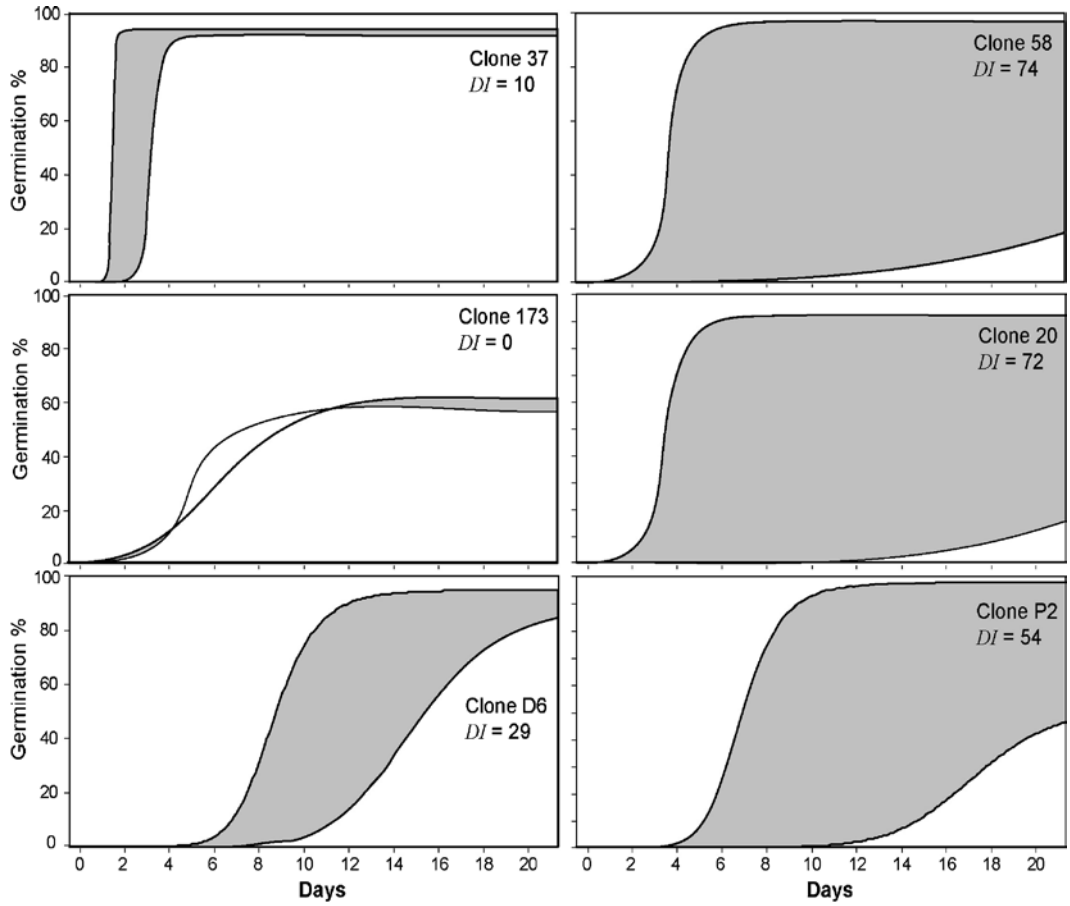

Fig. 1. A quantitative illustration of dormancy (DI) as determined by the area between germination courses of moist-chilled seeds (*upper line*) and untreated (non-moist-chilled seeds (*lower line*)) showing the range of variation among seed orchard clones of lodgepole pine (*upper panel*), white spruce (*middle panel*), and Douglas-fir (*lower panel*).

during germination. This is especially a consideration in northern climates, where greenhouse germination mostly takes place during early spring when outdoor temperatures are often below 0°C. Therefore, treatments that cause an increase in germination speed (and a decrease in the lag) have cost benefits for nurseries.

Due to the scaling up of the size of seed populations to be germinated when transitioning from the laboratory to the greenhouse, germination results may not be the same. The increase in space, number of seedlots, and species often results in environmental conditions being fairly heterogeneous; as a consequence, germination results often fall short of what was assumed based on laboratory germination testing. However, even under tight environmental control and following generally accepted standards, laboratory germination tests show that seedlot performance still can vary. For example, based on 3,173 germination tests, 1,798 seedlots of which were lodgepole pine, the average germination

capacity was 92% (27). Compounded by the much wider amplitude in environmental conditions in the nursery growing environment, such as soil medium, potential moisture gradient in the soil mixture, temperature differences in different locations within a greenhouse, and varying light intensity levels, the variation in germination parameters may be much larger than expected as compared to those based on laboratory tests. Furthermore, the seeding window in any nursery is much more prolonged or delayed due to operational constraints resulting in potentially more variation, especially in large seedlots. Finally, in the laboratory, a seed is scored as "germinated" when the radicle is four times the length of the seed; in the nursery, the assessment of germination is usually done much later (for example, when the cotyledons shed the seed coat) (11), adding another temporal component to the differences between laboratory and nursery measures of germination. Despite these contributing factors promoting the incongruence between laboratory and nursery results, in cases where this has been assessed, the differences between nursery and laboratory GC are small and account for only 2.5% across 17 tree species and 978 seedlots (28).

5.2. Bare-Root Nursery

The environmental conditions in bare-root nursery beds are not nearly as "controlled" as in greenhouses, and germination results are expected to vary considerably. With the exception of watering, the nursery manager cannot manipulate much of the outdoor environment to enhance germination. However, as it is not as crucial to have anticipated germination rates as predicted by laboratory tests, this may not present problems. However, it should be pointed out that the seed-to-seedling ratio in bare-root nurseries is expected to be much lower than that of their container counterparts. In a container nursery, every empty cavity affects the operation profit margin, since this space is heated, irrigated, and fertilized without any return on investment (see below). In a bare-root nursery, the practice of over-sowing to account for these short falls is more prevalent leading to the unwise use of seed which can be fairly serious, especially if high-quality seed, such as orchard seed, is used. Despite this, as noted below, container nurseries are not immune from this practice too. However, it has been shown that prolonged moist chilling renders seeds competent to germinate at (lower) temperatures than those that are more commonly encountered in uncovered nursery beds (29, 30).

6. Sowing to Maximize Germination Capacity

In container nurseries, the "space currency" is determined by the number of blocks used and the number of cavities per block. Thus, it is very important that each cavity within every block results in

the production of one seedling and this is more evident when blocks with larger cavities are used as relatively more space is allocated to each cavity (11). To counteract any fall-downs in the number of filled cavities, "over-sow" is commonly practiced. The amount of over-sowing is called the sowing factor and is precisely determined given the expected germination capacity of a seedlot (31). Of course, knowing the GC of a seedlot becomes crucial for achieving the objective of close to 100% cavity occupancy. This practice forces nursery managers to use a greater number of seeds per cavity as compared to the "ideal" one seed per cavity (which requires 100% germination capacity), and often leads to a waste of seeds.

The effects of improving germination capacity on the cost of nursery operations are illustrated here. The number of seeds required to produce one healthy and acceptable seedling at a germination capacity of 91% is 2.33. In comparison, at a GC of 98%, the number of seeds required drops to 1.87 (11). For a crop of 1 m^2 plantable seedlings, at a cost of 5 cents/seed, the difference is $23,000. Clearly, attempts to improve seed quality and germination percent have immediate pay-offs.

Multiple sowing usually results in the presence of multiple germinants per cavity, which is commonly followed by thinning after a certain period when germination is assumed to be complete. This practice not only wastes seeds, but can also have some undesired consequences as demonstrated by El-Kassaby and Thomson (19) and El-Kassaby (20). Since germination parameters (including dormancy, speed, and germination capacity) are under strong genetic control (maternal), it is expected that seeds from different seed donors (families) should manifest this genetic variation in their germination behaviour. Thus, certain families are preferentially thinned at an early stage if a greater proportion of their seeds are either affected by slow germination or deep dormancy. The resulting unintentional selection causes some families to be under-represented in the final seedling crop and in the most severe case, this practice could lead to the deployment of seedling crops with reduced genetic variability. If there is reduced genetic variability of orchards' seedlots and nursery crops, this may in turn lead to their diminished capacity to deal with unpredictable environmental contingencies during their long lifespan.

In bare-root nurseries, this unintentional selection may also take place but unless sowing is done in family blocks (i.e. using seeds from individual seed donors), there are no other remedies to counteract this negative selection effect. However, processing individual family seedlots may put too much strain on seed processing and seed storage facilities to be of a practical solution.

7. Seed Quality

To a large extent, the level of dormancy and the successful pretreatment to break it affect the onset (lag) and speed of germination. Seed quality on the other hand is the driving force governing germination capacity or maximum germination percent of a seedlot. In industrial settings, this is the most important attribute of a seedlot and strongly affects a multitude of cascading actions starting from the over-sow determination and ending with the expected size of seedling crops recovered at the end of the growing season. Of course, the role of dormancy cannot be overemphasized since standard seed pretreatments are insufficient to overcome deep dormancy and a seedlot's true germination capacity can be underestimated.

The proportion of non-viable seeds in a seedlot strongly affects the average seed quality and, as a result, the average germination capacity of a population. Because of this, it is most important to be able to reliably separate filled and viable seeds from non-viable and/or empty seeds (see separation procedures in ref. (11)). Seed production and mating dynamics should be investigated if the proportion of non-viable seeds is consistently from the same seed donor (for example, from a seed orchard). Causes for empty seeds could be high selfing rates, lack of pollen availability at the time of ovule receptivity, and insect predation.

The most reliable way to test for seed quality is to carry out a germination test under standard conditions using generally accepted protocols after recommended and prescribed dormancy-breaking treatments are applied. This testing procedure follows International Seed Testing Association (ISTA: http://www.seedtest.org) or Association of Official Seed Analysts (AOSA: http://www.aosaseed.com) rules which are designed for uniformity in testing standards and procedures. However, it should be pointed out that in several instances, the prescribed methods need further refinements (32, 33).

8. What Will the Future Hold?

Global warming and its associated climate changes are generally accepted in the scientific and forestry community. The extent of the impacts on forest communities and anticipated responses of forests are much more controversial and uncertain. One counteracting measure against the anticipated changes is a general trend to diversify the forests and possibly promote the use of hardwoods (broad-leaf trees) and southern conifers seed sources. Experience in the nursery industry is not advanced in growing broad-leaf trees as the bulk of the seedlings for regeneration in Canada is (still)

conifers. This implies that more research and testing are needed to develop suitable protocols and procedures to successfully grow poplars, alders, willows, birches, and maples on large commercial scales economically in forest nurseries as some species of these genera may present seed biology challenges (34). Additionally, the seed pretreatment of southern conifers seed sources might require some adjustments for attaining maximum seed use efficiency.

Finally, seeds from species with deep dormancy may not receive the required moist chilling at low temperatures if winters become warmer, shortening the time of chilling so that seeds may not germinate even under otherwise favourable germination conditions (35). However, Johnson et al. (36) have speculated that the "after-effect" phenomenon observed in Norway spruce (*Picea abies* L.) and other conifer species (37–40) is a regulating mechanism that affects adaptive plasticity rendering seedlings less susceptible to the potentially harmful effects of a rapidly changing environment. These types of studies point to an epigenetic mechanism (the precise nature of which is not yet known) that contributes to a rapid response to systemic changes in temperature (41). This mechanism operates during embryo development and adjusts the timing of bud set in accordance with the temperature conditions in which the mother tree lives. More specifically, the timing of dehardening and bud burst in the spring, leader shoot growth cessation in the summer, and bud set and cold acclimation in the fall are processes that are advanced or delayed according to the temperature during female sexual reproduction. Thus, seedlings (both normal seedlings and seedlings derived through somatic embryogenesis) "remember" the temperatures and photoperiod prevailing during embryogenesis and seed maturation. The epigenetic changes that potentially underlie this phenomenon clearly affect adaptive traits in Norway spruce and in other conifer species (40). More research is needed to understand the molecular basis of these phenomena, particularly events that underlie this type of "cellular memory" in plants.

Recently, genetic conservation efforts have increased and one aspect of conservation is ex situ conservation mainly in the form of seed collections stored in safe storage facilities. This conservation strategy relies on knowledge of optimal conditions during long-term seed storage, storability potential for each species, and known deterioration rates of seedlots (11).

9. Role of Molecular Biology and Genomics

Successful dormancy-breaking treatments and germination protocols have been worked out for most temperate forest species as demonstrated by the large number of seedlings produced and planted annually. However, there are recalcitrant species, where

more basic research is needed to elucidate the bottlenecks to achieve successful propagation at high germination capacity. Yellow cedar and some of the true firs (*Abies* spp.) present some challenges with respect to more complicated dormancy-breaking requirements and lower germination capacity (see Chapter 4). The work by Kermode and co-workers (e.g. (42); see also Chapter 4) is an example of how basic research is contributing directly to operational procedures.

The recent advances in gene expression research and genomics/functional genomics initiatives have created an opportunity for understanding the underlying biological basis of seed germination and dormancy. It is anticipated that the molecular studies on the most studied species, such as spruces and pines, will lead the way and provide a wealth of resources to further this area (e.g. through vast EST libraries and micro-arrays). Furthermore, QTL analysis (Chapter 11) further enhances our understanding of the genetic control over germination parameters.

Molecular studies may also contribute to elucidating the basis of the responses of conifers to changing climates. How do trees cope with stresses associated with seed production in an environment to which they are not adapted? Furthermore, as noted earlier, the molecular basis of the after-effects (cellular memory) should be studied, especially as it may become relevant to triggering adaptation mechanisms in a changing climate. Finding ways to increase the storability and reduce the aging process in stored seeds would also contribute to our management of seed, especially for the purpose of ex situ conservation.

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Chapter 2

Challenges Facing Seed Banks and Agriculture in Relation to Seed Quality

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Abstract

Seeds form a convenient vehicle for storage of germplasm, both for agricultural purposes and conservation of wild species. When required, seeds can be taken from storage and germinated, and plants can be propagated for the desired purpose, e.g., crop production or biome restoration. However, seed dormancy often interferes with stand establishment or industrial utilization in crops and germination of wild species. An anticipated termination of dormancy (i.e., before crop harvest) also occurs, with preharvest sprouting as a consequence. In order to overcome these problems, a better understanding of dormancy is required. This chapter is devoted to discuss the achievement of such understanding in problematic species.

Key words: Conservation, Crops, Seed deterioration, Seed dormancy, Preharvest sprouting, Seed storage, Wild species

1. Introduction

Seed dormancy has been a subject of intensive research during the last decades. Physiological and environmental control of dormancy has been studied in depth and, more recently, its molecular and genetic bases are starting to be elucidated. As in other areas of plant science, progress on the elucidation of the molecular basis of dormancy has been attained through the use of model organisms. Indeed, work with *Arabidopsis* has revealed the involvement of several genes controlling the different processes that converge to impose, maintain, or release dormancy. Beyond the fact that dormancy is a highly intriguing biological process and consequently moves the curiosity of scientists, the study of dormancy has strong practical implications. Both farmers and seed companies encounter problems associated with a lack of dormancy or conversely with profound dormancy. In the same way, industrial processes like malting, which depends on seed germination, can

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be largely hampered by the existence of dormancy or alternatively face important reductions in the quality of the received lots due to a precocious dormancy termination and pregermination in the field. Long-term seed conservation (i.e., seed banks) is also subjected to limitations imposed by dormancy and the paucity of knowledge that exists on the mechanisms behind the phenomenon.

Dormancy is a trait displayed by seeds of most temperate species. Since dormancy is highly undesirable for agricultural purposes, selection pressure against this trait must have been important throughout the domestication process, particularly in species of temperate origin. In some cases, this pressure must have gone too far, as in the case of cereals in which some crops are susceptible to preharvest sprouting (PHS) or pregermination, due to a loss of dormancy inception during seed development. Nevertheless, intraspecific variability for dormancy exists and "dormancy genes" are still present in some genotypes, although their positive selection through breeding has been difficult due to strong linkage to other undesired traits. In some other cases, selection pressure might have not succeeded in eliminating dormancy, so seeds are not germinable by the time of the next sowing or by the time they need to be "ready" by the malting industry. In any case, dormancy release should take place within a precise "time window." This rarely can be achieved without a solid knowledge of the mechanisms on which dormancy relies in each species. For this reason, a lot of research has been invested to elucidate dormancy mechanisms in problem crops (i.e., wheat, sorghum, sunflower, barley); some of these mechanisms are common to those of *Arabidopsis*, but others are not. With this in mind, most of the molecular information obtained from *Arabidopsis* and other model organisms has started to be "translated" into problematic species from a dormancy point of view. The latest achievements in the area come from this "translational" approach.

The role of seed dormancy is to spread germination across time, but in synchrony with the seasons to avoid unfavorable conditions for plant establishment (1). Different types of dormancy exist, first described by Nikolaeva (2) and more recently explored in detail by Baskin and Baskin (1). In seeds with *physiological dormancy*, a physiological mechanism prevents elongation of the axis, usually observed as radicle protrusion. Seeds with *morphological dormancy* are characterized by underdeveloped embryos that are either differentiated or not. Seeds with morphophysiological dormancy display a combination of morphological and physiological dormancy. In seeds with *physical dormancy*, the restricted permeability of water, typically caused by a palisade layer of lignified cells in the seed coat, interferes with imbibition and thus with germination. A combined dormancy of physiological and physical dormancy exists as well. This chapter focuses on physiological dormancy or the lack thereof.

In wild species that have not been cultivated, seed dormancy is often present (3). The type and degree of dormancy seem more pronounced in some families than in others. Dormancy forms a block to germination, which plays a role in the ecology of plant species. Dormancy is clearly an adaptive trait that contributes to the survival of plants by spreading germination over time, but in synchrony with the seasons, thus facilitating growth of the young plants and flowering when conditions are most favorable (1). Conservation of wild germplasm occurs by banking seeds under optimal storage conditions, often considered to be dry and at a subzero temperature, for long-term survival. However, only viable seeds should be stored under such conditions; dead seeds (for purposes other than conservation) are stored more cost-effectively at ambient conditions that require minimal infrastructure and maintenance costs. So far, a positive germination test has proven to form the most solid method to verify that stored seeds are viable. A germination test result is affected by any dormancy that is present, and any suspected dormancy must be released to get a proper estimate of the viability. These extra efforts require more resources: seed numbers, dormancy-releasing chemicals, space in germination chambers, a wider range of germination conditions, and more staff time to score germination and analyze viability. These are costs that cannot be entirely avoided, since wild species always display a degree of seed dormancy; but creating greater understanding of seed dormancy and the factors important for its termination can reduce them, and there is further a need for better tests to assess dormancy.

In this chapter, we point out the most important challenges facing agriculture and long-term seed conservation in relation to the presence or absence of dormancy. We present some examples of each type of problem, illustrating on the physiological mechanisms responsible for the expression of the character and commenting how the molecular information coming from studies on *Arabidopsis* and other model species have started to be used to attain the final aim of solving these dormancy problems.

2. Challenges Facing Agriculture

Seeds in their natural form are still the best way to preserve and propagate most crop species, and their capacity to germinate at the right moment (either in the field or under controlled conditions) is a most important feature to us. Therefore, any factors (internal or external) affecting the germination capacity of seeds and ultimately their agronomical performance should be understood in order to improve seed quality through breeding and/or crop

management strategies. Among the factors that may affect germination, dormancy is probably the most important, as it is a heritable trait that can inhibit germination of a viable seed.

Dormancy is the result of mechanisms operating within the seed that inhibit germination even when the seed is exposed to an environment known to be friendly for that species (i.e., in which water, oxygen, and adequate temperature are available). Although mechanisms behind dormancy seem quite diverse among different species, they converge at the point where cell expansion – that would result in embryo growth – is blocked. Primary dormancy is acquired during seed development. In most angiosperm species, primary dormancy is gradually lost after the seed is ready for dispersal (in wild species) or harvest during a postmaturation or after-ripening period. This process takes place in seeds during dry storage or in buried seeds in the soil as well, although these different environmental conditions can have great impact on the rate at which dormancy is terminated. Release of primary dormancy occurs at different rates depending mainly on the genotype and nothing else, but time is needed in many species for their seeds to be able to germinate (although some hard-coated seeds depend absolutely on chemical or mechanical scarification). Even in species that depend on the presence of light for germination (like many *Solanaceae*), sensitivity to light also varies with time after seed dispersal, and seed germination eventually becomes light independent. So the time lapse required by a seed population to lose dormancy and be able to undergo rapid germination immediately after sowing is a character that varies greatly not only between species, but also between different genotypes (i.e., cultivars, inbred lines, etc.) of the same species. The timing of the exit of dormancy for seeds of cultivated species can impact on agricultural systems in different ways.

2.1. The Persistence of Dormancy

For most cultivated species, the process of domestication has favored the abundance of genotypes that lose dormancy faster than their wild ancestors. A rapid and simultaneous germination of the seeds after sowing is required for a healthy and uniform stand of plants. A long-lasting dormancy (i.e., many months) would pose the problem of preventing a second sowing in the same season. Similarly, the process of malting relies on germination, and those genotypes that could be malted immediately after harvest are likely to be preferred. Nevertheless, among these reduced-dormancy genotypes, others with long-lasting dormancy have managed to survive up to the present and offer a source of variability for manipulating the level of dormancy through identification of dormancy QTL and its use in breeding programs. In species with a shorter history of domestication, like sunflower (*Heliantus annuus*), dormancy elimination has not been as dramatic as in cereal species and is frequently a problem in seed lots intended for sowing.

2.1.1. Sunflower: A Crop Species with Persistent Dormancy

Sunflower is a good example of a crop species with prolonged dormancy. Dormancy persistence in different sunflower genotypes can vary from several weeks up to almost a year. Also, important variation has been reported on this trait for the same genotype under different environments, indicating a strong interaction with the environment. Sunflower is cultivated in many areas around the globe, and is a summer crop. At harvest time, sunflower seeds are dormant and germinate poorly (4–6). This dormancy is the result of true embryo dormancy (4, 7) and the inhibitory action of the envelopes (4, 5, 7), including the seed coat and the pericarp since sunflower "seeds" are achenes. In the case of freshly harvested sunflower seeds, dormancy is expressed at temperatures lower and higher than 25°C (4). Dormancy expression at low temperatures is attributed to embryo dormancy which is not expressed at high temperatures (4); conversely, dormancy expressed at high temperatures results from coat-imposed dormancy (4). Consequently, a few weeks of dry after-ripening allow seed germination at low temperatures due to termination of embryo dormancy; the acquisition of the capacity to germinate at high temperatures, in contrast, may take several weeks of dry after-ripening (4).

The inception of embryo dormancy occurs relatively early during seed development. Sunflower embryos are germinable if isolated from the entire seed from as early as 7 days after pollination (DAP) and until approximately 12 DAP; the entire seed, however, germinates very poorly during this period showing that the coat (seed coat plus pericarp) imposes dormancy (Fig. 1) (4, 8).

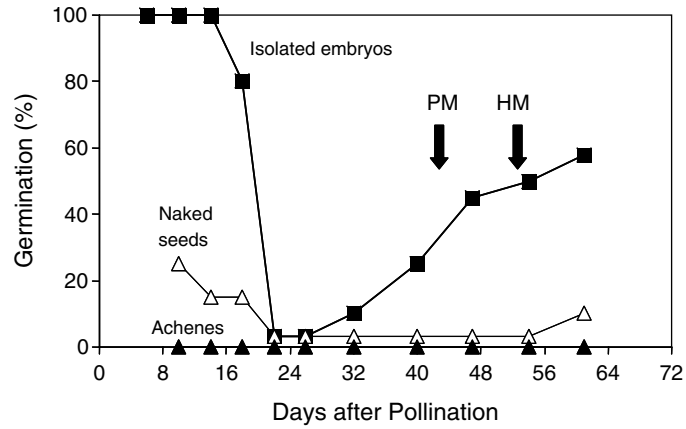

Fig. 1. Germination percentage of sunflower achenes (*solid triangles*), naked seeds (*open triangles*), and isolated embryos (*solid squares*) harvested at different days after pollination (DAP) and incubated at 25°C. Whole achenes are totally unable to germinate when incubated at any of the developmental stages displayed in the graph. The *arrows* associated with PM and HM indicate the approximate times during development when the crop reaches physiological maturity and harvest maturity, respectively. This figure has been redrawn with data originally published in LePage-Degivry and Garello (8) (Copyright American Society of Plant Biologists) and in Corbineau, et al. (4) (Permission of the Israel Journal of Botany). This Figure was first published in the "Handbook of Seed Physiology: Applications to Agriculture". Reprinted here with permission from the Haworth Reference Press of the Taylor and Francis Group.

From 12 DAP onward, embryo dormancy progressively develops and at 20–22 DAP, embryos are fully dormant (Fig. 1). This embryo dormancy is not eliminated if the axis is separated from the cotyledons, indicating that the axis itself is dormant (8). While the seed progresses toward maturation, embryos are slowly released from dormancy; by the time the grain has attained harvest maturity, some embryo dormancy still persists (Fig. 1). Therefore, the deep dormancy that sunflower grains present at harvest results from the coexistence of coat-imposed dormancy and some remnant embryo dormancy (4). Embryo dormancy is lost shortly after harvest if the seed is subjected to dry after-ripening, but coat-imposed dormancy persists for longer and may require several weeks of dry after-ripening to be overcome.

The plant growth regulator ABA appears to be involved in the imposition of embryo dormancy. The inclusion of fluridone (an inhibitor of ABA biosynthesis) in media upon which developing sunflower embryos are cultured prevents the induction of embryo dormancy (8, 9). Nevertheless, the pattern of accumulation of ABA in the developing embryo does not coincide with the physiological behavior of embryo: during seed development, embryos germinate well at the time when the endogenous ABA level is at its highest (7–12 DAP); thereafter, ABA decreases to a low value when embryo dormancy becomes established (9). It seems, then, that the ABA peak at early stages is responsible for the imposition of the dormant state that is established immediately after that peak has taken place.

Moreover, it appears that ABA needs to be present during a critical time period to induce dormancy. In an interesting study, LePage-Degivry and Garello (8) showed that when young (7 DAP), nondormant embryos are cultured in the presence of ABA, the hormone produces a temporary inhibition of germination but does not induce dormancy (i.e., embryos are able to germinate when transferred to a basal medium). In contrast, exogenous ABA becomes effective if applied immediately prior to the natural induction of dormancy. For example, a 5-day culture on a medium containing 5×10^{-5} M ABA results in dormancy of a proportion of 13 DAP embryos while induction of dormancy occurs in 100% of 17 DAP embryos. The authors concluded that either a change in sensitivity to ABA occurs during development or the existence of a second factor is necessary along with ABA to induce dormancy.

As mentioned before, embryo dormancy can be terminated by dry storage. Bianco, Garello, and LePage-Degivry (10) attempted to elucidate the mechanism through which dry storage terminates embryo dormancy by drying artificially dormant 17–26 DAP embryos and testing for germinability either immediately after drying or after leaving the embryos for 6 weeks in a desiccator (dry storage). A decrease in ABA content occurs immediately after the drying process which is not accompanied by a complete release

from dormancy and, on the other hand, additional dry storage does not affect the ABA content but promotes germination. In addition, the drying treatment also stimulates immature sunflower embryos and axes to respond to gibberellins (GAs) upon rehydration. Thus, although the drying treatment induces both a decline in ABA and an increase in sensitivity to GA, additional dry storage is necessary to obtain germination; the authors propose that dry storage suppresses the capacity of the embryo to synthesize ABA. The extent to which the drying treatment itself suppresses ABA biosynthesis was not examined. To identify the process by which dormancy is broken during after-ripening, Oracz et al. (11) focused on the role of reactive oxygen species (ROS) in this phenomenon. After-ripening is accompanied by a progressive accumulation of ROS, namely, superoxide anions and hydrogen peroxide, in cells of embryonic axes. This accumulation, which was investigated at the cellular level by electron microscopy, occurs concomitantly with lipid peroxidation and oxidation (carbonylation) of specific embryo proteins. Incubation of dormant seeds for 3 h in the presence of hydrogen cyanide (a compound that breaks dormancy) or methylviologen (a ROS-generating compound) also releases dormancy and causes the oxidation of a specific set of embryo proteins. In summary, the mechanism proposed by the authors involves ROS production and targeted changes in protein carbonylation patterns (11).

As with other cultivated species, such as *Lactuca sativa* (12) and *Arachis hypogea* (13), ethylene (C_2H_4) and ethephon strongly stimulate the germination of dormant sunflower seeds (4, 5, 14). In contrast, gibberellic acid (GA_3) and cold stratification (moist chilling) do not overcome dormancy in this species (15), although 1 mM GA_3 is effective for overcoming dormancy in some wild sunflowers (16). Corbineau et al. (4) showed that ethylene and its immediate precursor (1-aminocyclopropane-1-carboxylic acid) strongly stimulate germination of primary dormant sunflower seeds; on the contrary, inhibitors of ethylene (i.e., amino-oxyacetic acid and $CoCl_2$) or ethylene action (silver thiosulfate and 2.5 norbomadiene) inhibit germination of nondormant seeds. Beyond the evident practical implications, these results indicate that ethylene synthesized by the seeds themselves is involved in the regulation of sunflower seed germination.

Although endogenous ethylene is known to be involved in sunflower seed alleviation of dormancy, Oracz et al. (17) assessed the possible role of cyanide, which is produced by the conversion of 1-aminocyclopropane 1-carboxylic acid to ethylene, in this process. The beneficial HCN effect on germination of dormant embryos is associated with a marked increase in hydrogen peroxide and superoxide anion generation in the embryonic axes (17). The effect of HCN can be mimicked by the ROS-generating compounds, methylviologen and menadione, but suppressed by ROS

scavengers (18). The increase of ROS results from an inhibition of catalase and superoxide dismutase activities and also involves activation of NADPH oxidase. The expression of genes related to ROS production (NADPHox, POX, AO1, and AO2) and signaling (MAPK6, Ser/ThrPK, CaM, and PTP) is differentially affected by dormancy alleviation either during after-ripening or by HCN treatment; the effect of cyanide on gene expression may be mediated by ROS. Surprisingly, HCN and ROS both activate similarly ERF1, a component of the ethylene-signaling pathway (18).

The use of ethylene or its precursors appears as a promising technology to stimulate the germination of dormant sunflower lots. Possibly, seed companies have not adopted treatments based on these chemicals due to a lack of adequate devices to treat large amounts of seeds and increased costs associated with the treatments. Also, the correct doses may vary greatly with the genotype and the level of dormancy, and seeds can overreact to ethylene with adverse effects on the young plantlet known as the "triple response" (stunting of growth, twisting of plants, and abnormal thickening of stems). The effect of storage conditions is being explored as it has been observed that high temperatures (i.e., 25°C) accelerate dormancy release of sunflower seeds as compared to storage at 5°C.

2.2. Cereal Dormancy: A Two-Edged Sword

2.2.1. Persistence of Dormancy in Malting Barley

In addition to impairing a rapid and simultaneous germination after sowing, a persistent dormancy would prevent the utilization of a seed lot for industrial purposes when the process requires germination. This is the case for the malting process, which uses barley as the main grain. The malting process itself requires grain germination, so a low dormancy level at harvest is a desirable characteristic because the grain can be malted immediately after crop harvest, thus avoiding costs and deterioration resulting from grain storage until dormancy is terminated. Therefore, breeders have to solve the compromise between the desire to obtain genotypes with low dormancy at harvest, but without the worry that dormancy is so reduced as to risk sprouting (a phenomenon which is referred to in the next section). In other words, dormancy release in barley must be adjusted to occur within a narrow and precise "time window." The achievement of this aim requires a thorough knowledge of the mechanisms determining dormancy release in the maturing grain.

Dormancy of the barley grain is typically imposed by the seed-covering structures (lemma and palea, pericarp plus seed coat). Indeed, embryos can germinate well from the very early stages of development if they are isolated from the rest of the grains and incubated in water (19). Limitation of oxygen supply to the embryo by oxygen fixation as a result of oxidation of phenolic compounds in the lemma and palea (hereafter referred to as the glumellae or the hull) has been suggested to be responsible for the dormancy of dressed caryopses of cereals, such as barley (20) and oat (21).

In dormant grains of barley, for example, whole intact caryopses germinate with difficulty, even in the air, while dehulled caryopses are all able to germinate under oxygen tensions of at least 10%, suggesting that oxygen concentration under the covering structures might be less than 10% (20).

As in many other species, dormancy in barley can be regarded as a relative phenomenon, the expression of which depends on the incubation temperature. It is usually not expressed at relatively low temperatures (10–20°C) while its expression increases as the temperature rises (20, 22, 23). The amount of water in the incubation medium also allows differential expression of dormancy in barley grains. Indeed, most barley cultivars, which present some dormancy at harvest, do not germinate if the grains are incubated in a Petri dish at favorable temperatures but with 8 or even 6 mL instead of 4 mL of distilled water (24); the same does not occur in grains from cultivars with low dormancy or in those that have after-ripened, showing that it is truly an expression of dormancy. This phenomenon is known by the malting industry as "sensitivity to water" and is one of the quality parameters assessed upon reception of a grain lot. This sensitivity to water is likely related to oxygen deprivation (hypoxia) imposed by the presence of the hull, which may be enhanced by an excess of water in the incubation media.

Dormancy of the barley grain also appears to be under ABA control: termination of glumellae-imposed dormancy during grain development correlates with a sharp decline both in ABA embryonic content and sensitivity (19). A role for ABA in dormancy maintenance of the barley grain has also been suggested: the ABA content of embryos declines during the first hours of incubation of nondormant seeds, whereas it remains at high levels in embryos of dormant grains (25). In recent years, Benech-Arnold et al. (26) confirmed this role of ABA in dormancy maintenance and extended it to the differential expression of dormancy at different temperatures. The ABA level of the embryo largely decreases during the first hours of incubation and prior to any visible germination in both dormant and nondormant grains at 20°C, the temperature at which dormancy is not expressed, and in nondormant grains incubated at 30°C, suggesting that ABA catabolism and/or conjugation exceeds ABA biosynthesis. By contrast, at 30°C, dormancy expression is associated with maintenance of ABA at high levels: after an initial increase, ABA content decreases very smoothly and is always between two- and fourfold higher than in embryos from grains in which dormancy is not expressed (Fig. 2). Millar et al. (27) examined gene expression profiles in *Arabidopsis* focusing on the gene families corresponding to ABA biosynthetic and catabolic enzymes, i.e., the 9-cis epoxycarotenoid dioxygenasegene and ABA 8'-hydroxylase gene families (CYP707A), respectively. Of these, only the *AtCYP707A2* gene was differentially

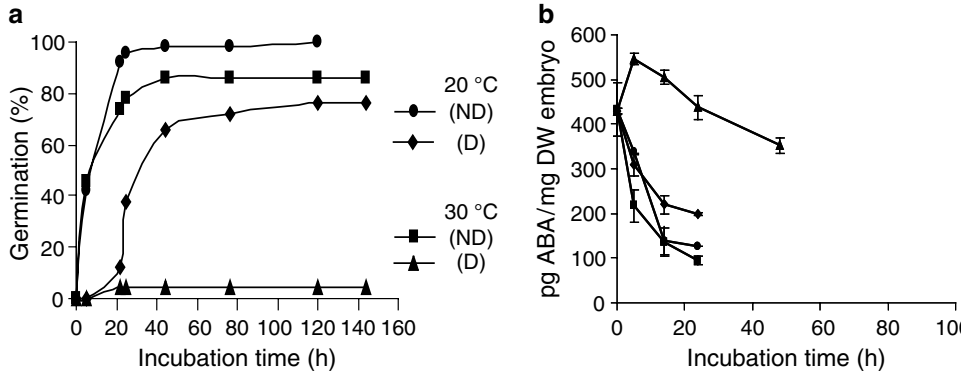

Fig. 2. Germination (**a**) and embryo ABA content (**b**) during incubation at 20°C (*circles, diamonds*) and 30°C (*triangles, squares*) of dormant (*circles, triangles*) and nondormant (*diamonds, squares*) grains. Grains were either freshly harvested (*dormant*, *D*) or were stored dry for at least 3 months at 25°C (*nondormant*, *ND*). Determinations are the means of two measurements ± arithmetical spread (germination) or of three measurements ± SD (ABA content). This figure has been redrawn with data originally published in Benech-Arnold et al. (26) and was printed with permission of Oxford University Press.

expressed between dormant and nondormant seeds, exhibiting higher expression in nondormant seeds. Similarly, a barley *CYP707* homologue (*HvABA8'OH-1*) was expressed to a much higher level in embryos from nondormant grains than from dormant grains (27). Consistent with this, in situ hybridization studies showed that *HvABA8'OH-1* mRNA expression was stronger in embryos from nondormant grains. Surprisingly, the signal was confined to the coleorhizae, suggesting that this tissue plays a key role in dormancy release (27). These results support a role for ABA in dormancy maintenance of the barley grain, and further indicate that dormancy release may be partly mediated by ABA catabolism via the 8'-hydroxylation pathway.

Both the removal of the glumellae and the incubation of dehulled grains under low oxygen concentrations modify grain germination behavior and ABA content evolution throughout incubation at 30°C (26). Thus, the glumellae is instrumental for dormancy maintenance because it imposes oxygen deprivation to the embryo, which, in turn, promotes ABA synthesis and/or inhibits ABA inactivation. Indeed, removal of the glumellae in dormant grains incubated at 30°C under 21% oxygen suppresses the initial increase in ABA content that occurs in dressed dormant grains incubated at the same temperature. By contrast, incubation under hypoxia (5% oxygen) of dehulled grains restores it and inhibits germination (26). In a recent paper, Mendiondo et al. (28) investigated the effect of the glumellae on the expression of ABA synthesis and catabolism genes, and the extent to which artificially imposed hypoxia can mimic the effect of the glumellae on gene expression. The presence of the glumellae enhanced, in dormant grains incubated at 30°C, the expression of genes encoding enzymes involved in ABA synthesis (HvNCED1 and HvNCED2)

without altering substantially the expression of HvABA8′OH-1. These results indicate that the presence of the glumellae maintains dormancy through an enhancement of ABA synthesis regulated at the level of gene transcription. On the other hand, artificially imposed hypoxia does not mimic the effect of the presence of the glumellae in terms of alteration of gene expression, thus casting doubt on the contention that the glumellae effects (at least on the expression of ABA synthesis genes) are through oxygen deprivation. Only the expression of HvABA8'OH-1 is enhanced by hypoxia, possibly as a feedback effect that results from poor functioning of the enzyme at low oxygen tensions (28).

Artificially imposed hypoxia also enhances embryo sensitivity to ABA by several folds (26). These results suggest that, in addition to interference with ABA metabolism, the presence of the glumellae increases embryo responsiveness to the phytohormone. To explore this possibility, Mendiondo et al. (28) measured the expression of several components of the ABA-signaling pathway during incubation of entire and dehulled grains: the presence of the glumellae enhanced the expression of most of the investigated genes, suggesting that the hull increases the sensitivity to ABA of the enclosed embryos and that this enhancement is effected at the level of gene expression. Again, artificially imposed hypoxia is not able to mimic the effect of the glumellae on the expression of ABA-signaling components, indicating that the apparent enhancement of embryo sensitivity to ABA under low oxygen tensions is due to overaccumulation of ABA as a result of malfunctioning of the enzyme committed to ABA inactivation (26).

One of the most popular methods used by the malting industry, whenever allowed by the customer, is the addition of gibberellic acid to the incubation medium to promote the germination of dormant barley grains. Indeed, it is well-known that gibberellic acid at low concentrations (0.1–0.2 ppm) stimulates germination of these grains (29). Studies on the most appropriate point in the malting process at which to add gibberellic acid have concluded that it should be sprayed on soon after the grain is removed from the steep (29). Other methods to remove dormancy in barley include the use of dilute solutions of hydrogen sulfide and keeping the grains for 3 days at 40°C, either in the open air when their moisture content falls to about 8% or in closed vessels when moisture contents are unchanged at between 17 and 20% (24). However, when the customer allows none of these methods, time of after-ripening is the only way of obtaining a germinable lot. Unfortunately, the physiology and molecular biology of after-ripening in barley has not been assessed as it has been in sunflower. Only experiments comparing dormant and nondormant grains have been reported in the literature as commented on in the above paragraphs. Within this frame, Gubler et al. (30) found that after-ripening has no effect on expression of ABA biosynthesis genes,

but promotes expression of an ABA catabolism gene (*HvABA8'OH1*), a GA biosynthetic gene (*HvGA3ox2*), and a GA catabolic gene (*HvGA2ox3*) following imbibition. In the same paper, the authors report that blue light mimics the effect of white light (which promotes dormancy) on germination, increasing ABA levels, and expression of GA and ABA metabolism genes (30).

2.2.2. The Lack of Dormancy in Cereals: Preharvest Sprouting

The advantages of having a freshly harvested seed lot with the capacity to germinate rapidly and uniformly under a wide range of environmental conditions are related to the possibility of immediate sowing or malting, thus avoiding financial costs associated with delays and/or the need for storage until germination capacity is good enough. Nevertheless, genotypes that produce nondormant seeds at harvest may already be able to germinate to some degree even before harvest. The main problem related to an early loss of dormancy in crop species is PHS. This phenomenon is a characteristic of cereal species, like rice, barley, wheat, and sorghum. As these species all exhibit intraspecific variability for the rate of dormancy loss and PHS behavior, genotypes with contrasting sprouting behavior have proved useful for many comparative studies (31–34), in addition to QTL analysis to identify several loci related to dormancy (35–38). When low levels of dormancy during late grain maturation period are combined with rainy or damp conditions in the field, the process of germination is activated while the seeds are still attached to the mother plant, and the resulting emergence of the radicle from the seed coats is called PHS. Depending on the intended purpose for the seeds after harvest, PHS can have serious negative consequences on their quality and this is economically penalized by the industry. PHS in cereals is a problem in many parts of the world and in some wheat production areas, PHS occurs in 3–4 years out of 10. Total worldwide annual losses have been estimated to about US$1 billion, but precise statistics are lacking. Direct economic losses caused by PHS to producers occur in several ways: desiccation of a sprouted grain leads to its subsequent loss of viability because, together with the activation of metabolism implicated in embryo growth, tolerance to dehydration is lost rendering the sprouted grain useless for sowing or malting. Sprouting also promotes carbohydrate respiration that not only reduces grain yield, but also creates a favorable environment for the attack of grains by saprophytic fungi and bacteria that produce toxins.

Depending on the level of dormancy and environmental cues, such as water availability for imbibition and temperature, the germination process can advance to different extents, and grains do not always reach the completion of germination and sprouting (i.e., germination is complete when embryo growth begins, and postgerminative growth leads to visible radicle and/or coleoptile emergence through the seed-covering structures). Even when desiccation occurs before germination is complete, starch degradation

may have advanced partially. This phenomenon is known as pregermination. Pregerminated grains cannot be distinguished visually, but the level of starch degradation is correlated with a decrease in falling number (FN) values (31) and a reduction in seed lot longevity (39). Industrial processes based on wheat and barley (flour and malting) are particularly sensitive to sprouting and pregermination. Seed lots are commonly assessed with the FN test, with smaller FN values indicating a greater degree of starch degradation. Wheat and barley seed lots can be penalized or even rejected if FN values show moderate incidence of pregermination and sprouting.

The phenomena of PHS and pregermination are closely related with the process of domestication. Cereal crops have a brief period of dormancy as compared to their wild ancestors. Throughout many years, farmers have pressed toward the selection of low levels of dormancy along with other traits, such as nonshattering, increased grain size, and less pigmentation. The occurrence of PHS not only depends on morphological and physiological traits genetically controlled (such as infructescence structure and permeability of structures surrounding the seeds and seed dormancy), but also depends on environmental factors (water availability and temperature). A single genotype may express PHS when grown in some areas, but not in others. Seed dormancy is the main heritable factor that contributes to PHS resistance, but the many attempts to control it through breeding programs have shown that dormancy is tightly linked to other interesting traits. Breeding programs that attempted to separate characters associated with seed color, dormancy, and longevity suggest that these characteristics may not always be separable and are referred to as domestication block (40). For example, in rice, both loci sh-h (for shattering) and Rc (conferring red pericarp) are tightly linked together with a QTL qSDs-7-1 for seed dormancy, implying that this region might represent a domestication block in the evolutionary pathway of rice (41). A mutation in the R-gene in white wheat is required for low tannin content (higher flour quality), but is also responsible for lower levels of dormancy (low PHS resistance). However, grain dormancy is a complex trait that relies on numerous genes, and other QTLs contributing to dormancy that are not linked to seed color have been identified and are being considered in breeding for PHS tolerance in white wheat (42).

In addition to classical breeding techniques, elucidation of the mechanisms involved in the control of dormancy may open other possibilities for manipulation of dormancy through genetic engineering techniques. Cereal species, like barley and sorghum, have served as model systems to study the mechanisms behind dormancy with the objective to understand the hormonal basis of the repression of germination in barley and sorghum, especially related to hormone metabolism and signaling (19, 20, 26, 32–34, 43).

2.2.3. Problems Associated with Preharvest Sprouting in Sorghum

Sweet sorghum (*Sorghum bicolor*) domestication began in Africa and later spread to India and the rest of the world. As in other cereal species, sorghum has a great intraspecific variability for the duration of dormancy: in some genotypes, dormancy may last up to several months while in others dormancy release may begin even before physiological maturity is reached. Due to its C4 metabolism, sorghum is often cultivated in hot, dry regions where other crops would fail. But recent introduction of sorghum to more humid cultivation areas has made this crop susceptible to PHS. Studies using two inbred lines with contrasting sprouting behavior (IS9530, PHS resistant, and RedlandB2, susceptible) have shown that dormancy release in the susceptible line begins between 20 and 30 DAP (Fig. 3). Dormancy in these sorghum lines is coat-imposed, and isolated embryos are capable of germinating fully from early development regardless of the genotype and the level of dormancy of the whole grain (Fig. 3). Previous research with both lines has shown that dormancy is established during early stages of seed development and is regulated mainly by the antagonistic effects of plant hormones, ABA (which enhances dormancy and inhibits germination) and gibberellins (which reduce dormancy and promote germination) (32, 33). However, absolute levels of both hormones in developing sorghum embryos are not related to the different level of dormancy exhibited by both sorghum lines.

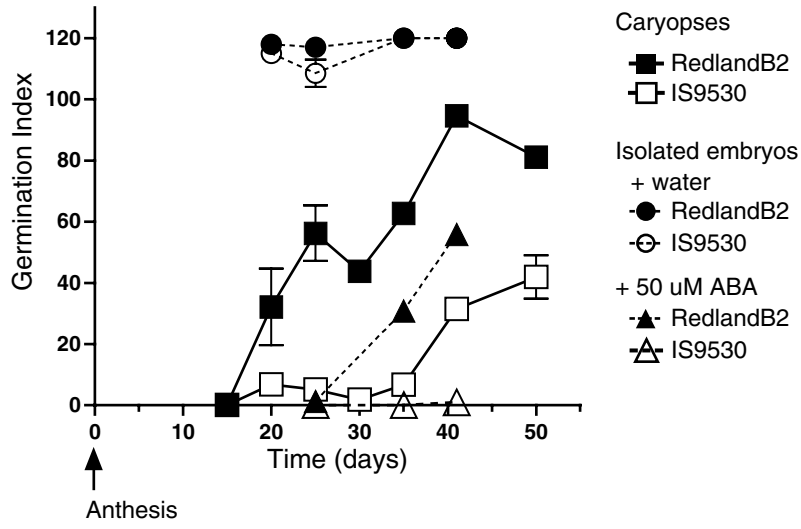

Fig. 3. Germination index for sorghum lines IS9530 and RedlandB2. Caryopses were harvested at different days after pollination (DAP) and incubated for 12 days in water at 25°C (*squares*). Embryos (axis plus scutellum) were dissected and incubated at 25°C in distilled water (*circles*) or 50 μM ABA (*triangles*). Each data point is the mean value of two biological replicates (i.e., two field plots) assessed in triplicate. *Bars* indicate standard error with $n = 2$. The approximate time of physiological maturity is indicated with an *arrow*. This figure has been redrawn with data originally published in Rodríguez et al. (34) and was printed with Permission of American Society of Agronomy, Inc., Madison, WI, the USA.

Nevertheless, different responses can be explained by sensitivity to these hormones and/or their postimbibition metabolism. Incubation of isolated embryos in a solution of ABA inhibits germination in a way that resembles the dormant grain, and an overall correlation between changes in grain dormancy and embryo "sensitivity" to ABA can be observed in both sorghum lines throughout development (32) (Fig. 3). In contrast to other species in which ABA accumulates in imbibed dormant seeds, ABA levels in these sorghum lines decrease at a similar rate and are not related to the level of dormancy.

In accordance to the observed contrast in embryo sensitivity to ABA, several sorghum genes known to be involved in ABA signaling in other species of reference are expressed differently in imbibed sorghum grains with contrasting levels of dormancy (34) (Fig. 4). Genes encoding several transcription factors (*SbVP1*, *SbABI4*, and *SbABI5*) and a protein kinase (*SbPKABA1*) that are positive regulators of ABA signaling are induced transiently in dormant IS9530 (PHS resistant) immature grains (just before physiological maturity), and may be involved in the higher sensitivity to ABA in this line. No induction of these genes is observed in the PHS susceptible line. These observations were also confirmed at the protein level for SbABI5. Nevertheless, expression of these genes (and ABI5 protein levels) fails to explain differences in grain dormancy and embryo sensitivity to ABA after physiological maturity, suggesting that mechanisms involved in the control of germination by ABA may differ with the developmental stage of the seed (before and after physiological maturity). This is in accordance with a biphasic pattern of dormancy release observed in sorghum and other species (i.e., barley, see next section).

On the other hand, ABA inhibition of germination is opposed by the germination-promoting effect of gibberellins. The ABA/GA balance governs imposition of dormancy during early development (33) as well as the expression of dormancy in imbibed grains. Quantification of embryonic GA in imbibed immature sorghum grains shows that embryos belonging to the PHS susceptible line RedlandB2 produce higher levels of active GA (GA1, GA3) as compared to those from the dormant line IS9530. This is correlated with a lower expression of a gene encoding a GA inactivation enzyme (GA 2-oxidase) in the nondormant line RedlandB2 while expression of this gene is higher in dormant IS9530 grains (Fig. 4). Thus, less-dormant grains are able to produce higher GA levels, in addition to the different ABA signaling activities. The observation that ABA inhibits an increase of GA levels in RedlandB2-isolated embryos suggests the existence of a mechanism by which ABA activity interferes with GA metabolism either by inhibiting GA biosynthesis or promoting GA catabolism. A possible link between enhanced ABA signaling and the promotion of GA catabolism activity through transcriptional activation of GA 2-oxidase genes is currently under investigation.

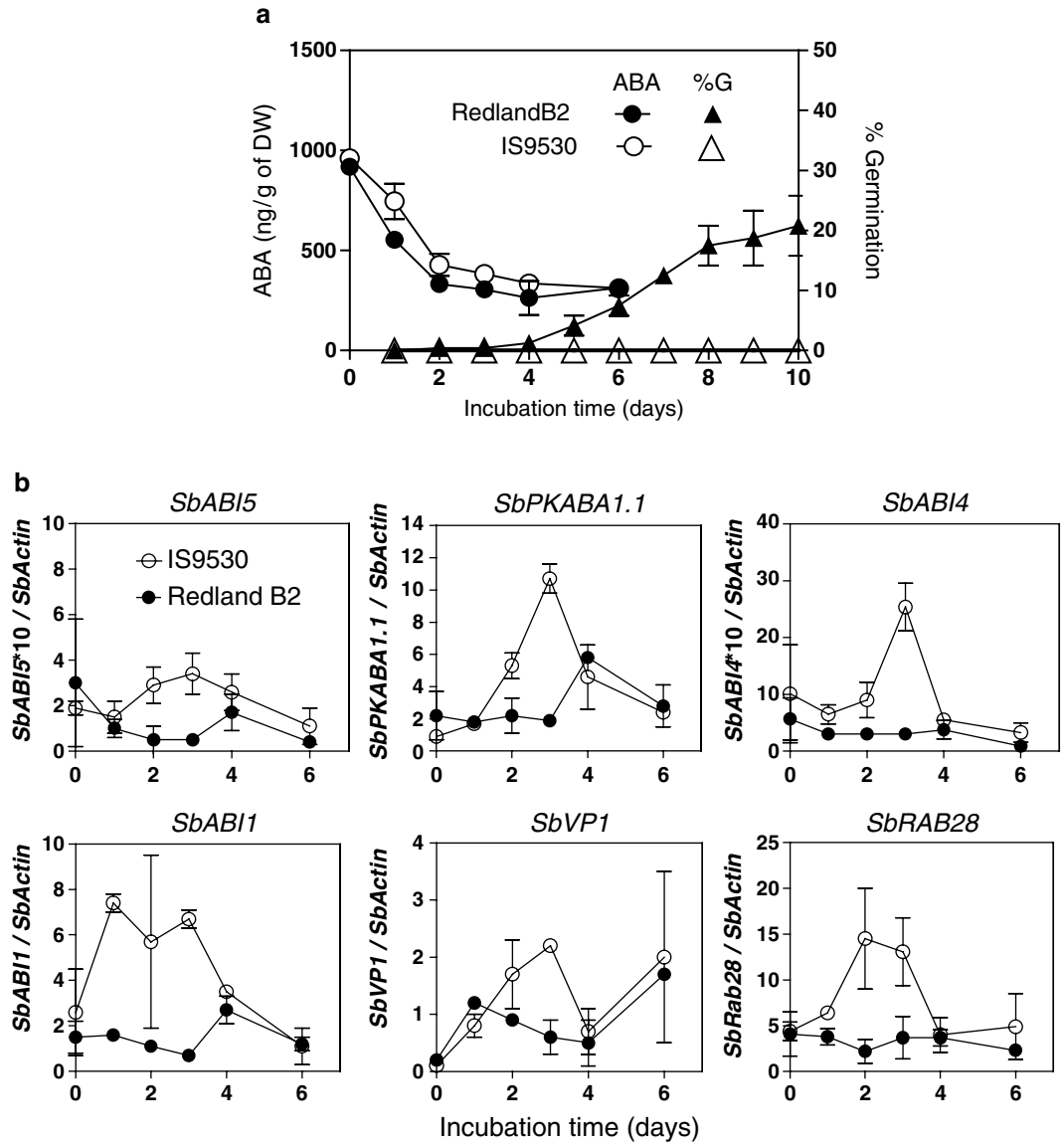

Fig. 4. (**a**) ABA embryonic content and germination response during incubation at 20°C of sorghum caryopses (30 days after pollination) from two inbred lines: sprouting resistant IS9530 and sprouting susceptible RedlandB2. (**b**) Expression (determined by RT-QPCR, relative to SbActin) of sorghum genes encoding homologues for ABA-signaling proteins: transcription factors SbABI4, SbABI5, SbVP1, protein kinase SbPKABA1, and a PP2C-type protein kinase, SbABI1. SbRAB28 was included as an ABA-responsive gene. Each value is the mean of two biological replicates coming from two independent field plots. This figure has been redrawn with data originally published in Rodríguez et al. (34) and was printed with permission of Oxford University Press.

2.3. Environmental Control of Dormancy

The duration of dormancy is determined mainly by the genotype but, as in many other species, it has been known for long that dormancy in grain crops can also be influenced by the environment experienced by the mother plant (44–48; see also 49 for references). Indeed, the effects of the parental environment on seed

dormancy have been reported for a wide range of species (for reviews, see 50, 51). Some well-defined patterns emerge, however, with certain environmental factors tending to have similar effects on different species. For example, low dormancy is generally associated with high temperatures, short days, drought, and nutrient availability during seed development (50, 52–55). The assessment and quantification of these effects might lead to the development of predictive models that could be of great help for reducing the incidence of problems associated with either reduced or persistent dormancy in crop production.

Among the different factors acting on the mother plant, temperature appears to be a major cause of year-to-year variation in grain dormancy of a same genotype. This is the case with barley, and different works have shown that temperature is effective in modulating the pattern of dormancy release only within a sensitivity period during grain filling (46, 56–58). A first predictive model was developed by Reiner and Loch (46), who determined that low temperatures during the first half of grain filling, combined with high temperatures during the second half, result in a low dormancy level of the barley grain and presumably in PHS susceptibility. The authors established a linear relationship between the ratio of the temperatures prevailing at both halves of the filling period and the dormancy level of the grains 3 weeks after harvest. This model was since used by the German malting industry to predict dormancy levels in the malting barley harvest lots. In a more recent work, Rodríguez et al. (58) identified a "time window" within the grain-filling period of cultivar Quilmes Palomar, with sensitivity to temperature for the determination of dormancy. The grain-filling period was divided in discrete intervals within a thermal timescale ranging from anthesis to physiological maturity (430°C days, accumulated over a base temperature of 5.5°C). This "time window" was found to go from 300 to 350°C days after heading. A positive linear relationship was established between the average temperature perceived by the crop during this "time window" and the germination index of the grains 12 days after physiological maturity (Fig. 5a, b). Twelve days after physiological maturity is approximately half way between physiological and harvest maturity; grain germination index measured at this stage is a good estimate of the rate with which the grains are being released from dormancy after physiological maturity. In addition, dormancy release shows a biphasic pattern with a short plateau between 10 and 14 DAP, in which the GI value remains relatively stable (Fig. 5a). According to this model, the higher the temperature experienced during the sensitivity "time window," the faster the rate with which grains are released from dormancy after physiological maturity and, consequently, the lower the dormancy level prior to crop harvest. Such a situation, combined with a forecast of heavy rains for the forthcoming days, implies a risk for the crop and the farmer could decide

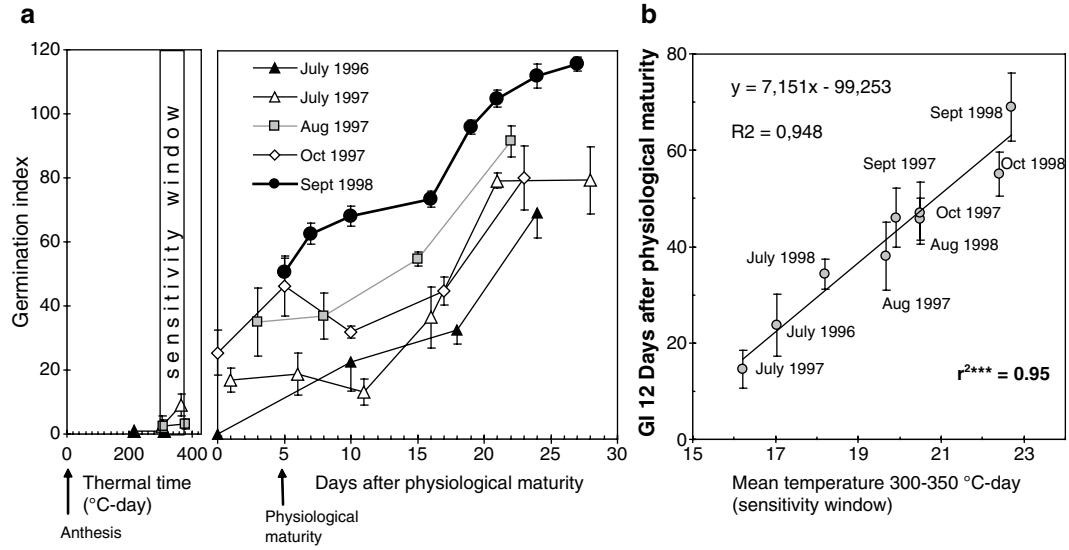

Fig. 5. (**a**) Germination index for barley grains (Quilmes Palomar CV) harvested at different moments after anthesis. A thermal timescale was used to describe the anthesis–physiological maturity period to compare grain-filling stages of plots grown under different thermal environments (base temperature for grain-filling period: 5.5°C). After physiological maturity, a chronological scale was used. Quilmes Palomar CV was sown on different dates during 1996, 1997, and 1998. Each value is the average of three subplots. *Vertical bars* indicate SE. (**b**) A linear equation was used to describe the relationship between the germination index of grains harvested 12 days after physiological maturity and incubated at 20°C and mean air temperature values obtained within the 300–350°C·day interval after anthesis for Q. Palomar cultivar sown on nine dates between 1996 and 1998. Regression equation: GI12DAPM = 7.14 × (Tm300–350) – 98.8 ($r^{2***} = 0.95$, $P < 0.0001$, $n = 9$). *Vertical bars* indicate SE. This figure has been redrawn with data originally published in Rodríguez et al. (58) and was printed with permission of Oxford University Press.

to anticipate the harvest. Conversely, low temperatures experienced by the crop during the sensitivity window would result in a high dormancy level prior to harvest, making the crop resistant to sprouting. This model was successfully validated against data collected from commercial plots 700 km away from the site, where the model was produced (58) and later validated with other commercial cultivars (59). However, it was also noted from validation that temperature explains only one dimension of the observed variability in dormancy. Indeed, there appear to be other unknown factor(s) that are responsible for displacing up or down the relationship between temperature and dormancy (58). Current efforts are directed to identify these factors and to quantify their effects. Notably, water stress promotes dormancy release in several barley cultivars (60), but this effect is noticeable only under moderate temperature conditions; in warmer environments, dormancy is low enough and masks any effect of water stress.

Sunflower is an exception to the general rule that low dormancy is associated with high temperatures experienced during grain filling. In this species, high temperatures during grain development result in an extended period of dormancy (61). In this case, grains that develop at high temperatures require a longer time

of dry after-ripening to acquire the capacity to germinate at low temperatures. Since embryo dormancy is expressed at low temperatures, it might be, then, that high temperatures during grain filling extend the duration of embryo dormancy. Germination at high temperatures, however, was not tested, and therefore it is not possible to say whether the duration of coat-imposed dormancy was also extended by high temperatures during grain development. When plants of sunflower commercial hybrids are exposed to 32°C (control) or 38°C (treated), seeds of the latter exhibit prolonged dormancy. In this case, germination was tested for both achenes (at 25°C) and isolated embryos (at 11°C) (Bodrone, unpublished results). Surprisingly, embryo dormancy was diminished by previous exposure to high temperature while the achenes behaved in the opposite manner. These results indicate that the enhancement of dormancy by high temperature during fruit development is related to coat-imposed dormancy, and the importance of this effect overrides the simultaneous reduction in embryo dormancy. Nevertheless, more trials are still needed to obtain a predictive model as in the case of barley.

3. Challenges Facing Seed Banks

Seed dormancy is usually lost in the process of cultivation. One likely explanation for this phenomenon is the removal of a selection mechanism by the farmer by controlling the time of germination through storing dry seeds and sowing in the soil at the appropriate time of year. However, as pointed out above, strong linkage with other traits can hamper the elimination of dormancy in selection and breeding. This ancient farm management tool has diminished the requirement for seeds from arable crops to sense the correct time of year for germination. Selection for reduced dormancy has occurred traditionally as only those seeds that germinate quickly and contribute to the seed crop are maintained in the farmer's seed bank. In this agricultural context, survival of the fittest seeds is established through a correct dose of dormancy, i.e., low enough to allow untroubled germination but high enough to prevent vivipary. For the correct timing of plant growth and survival in the field, dormancy is no longer required. Dormancy in most cultivated and bred crops is lost, which indicates that it is an expensive trait that is redundant if it no longer provides a function or advantage. On the other hand, this does not apply to many wild species that still require a sensing mechanism to complete germination in the correct time of year. Consequently, when working with wild species, all types of dormancy should be anticipated, particularly when these are members of families that are frequently troubled (3). The presence of dormancy imposes problems in the

correct assessment of seed quality, as it can cause an underestimate of the viability. Knowledge of dormancy is, therefore, important.

Seeds that do not complete germination are either dormant or dead. Usually upon maturity, seeds are shed with high viability. Seed viability decreases depending on the stage of maturation at harvest, the environmental conditions, and the time spent at these conditions. Temperature and water content play an important role in maintaining seed viability. Generally, for orthodox seeds, a lower temperature and lower water content help to keep high seed viability for a longer time. The seed longevity for each species influences how long high viability can be maintained. There are reports of unusually high seed longevity, e.g., for sacred lotus (62). However, many species display a much shorter longevity, and even longevity as short as 1 year has been reported for an orthodox species despite optimal storage conditions (63, 64). The intraspecies variation in genetic background contributes to seed longevity, and it is likely that interspecies genetic variation for these (and other) loci plays a similar role (65). Strongly reduced longevity is also reflected in recalcitrant storage behavior of seeds that are intolerant to desiccation and, therefore, cannot usually be stored for extended periods of time (66). Moist storage of these seeds leads either to germination or death within a fairly short time span. Cryostorage is an alternative to dry storage, but not all recalcitrant seeds are suitable for cryostorage. The need for development of protocols for both freezing, e.g., in the presence of cryoprotectants, and recovery from cryostorage combined with higher costs for cryopreservation makes this technique less suitable for widespread application. Vivipary is an extreme form of reduced longevity, since these seeds display precocious growth while still attached to the mother plant, making storage impossible. This phenomenon is the natural counterpart of dormancy represented in crops through PHS, as pointed out above. Similar to dormancy forming a challenge for seed banking, vivipary does so too. True vivipary has been mainly encountered in species populating shallow marine habitats with a highly reduced chance for offspring to disperse to a patch that is different from the parent's habitat, thus eliminating the need for a selection mechanism, like seed dormancy (67). Although true vivipary is distributed to a relatively limited extent in the plant kingdom, knowledge of the topic not only contributes to the understanding of dormancy, but also PHS in crops.

Seeds stored dry in seed banks inevitably lose viability as a result of storage time and/or suboptimal conditions. However, any dormancy that is present is not usually affected by these conditions. As a result, seed viability undergoes a reduction while dormancy is maintained during dry storage, leading to increasing difficulties with germination upon dry storage. Dry after-ripening releases dormancy of *Arabidopsis* seeds and a molecular pathway has been described (68). While dry after-ripening is typically

displayed at elevated temperatures and water contents, it is not at subzero temperatures and low water contents. Dry after-ripening is also a characteristic of nondeep physiological dormancy, but not of other types of dormancy (69). Therefore, during storage under optimal conditions, dormancy is not expected to be released, and seed quality is expected to decline as a result of a reduction in viability. Seed storage inevitably leads to an increase in problems with germination. A positive viability test becomes progressively harder to accomplish with longer storage time.

The purpose of seed banks is to store seeds with high viability as long as necessary. Testing the quality is essential to serve the higher goal of seed banks, which is to preserve the germplasm for future use. Such would be true for any seed bank; but in the case of wild germplasm seed banks, e.g., the Millennium Seed Bank, an additional goal is to conserve species and protect them from potential extinction through ex situ conservation. Dormancy interferes with viability testing, but it is necessary that the test discriminates between dormancy and viability. Limiting the number of seeds required for a test would also be desired to avoid wasting precious materials. When applying conventional germination tests, large seed numbers are often required for an accurate assessment since different dormancy release factors can be used. Dormancy in situ is often released by environmental factors, since it forms a mechanism that links germination to the correct season. For many species, temperature is an important factor that determines dormancy release. The temperature requirements for dormancy release often differ from those for germination. Seeds of many species with physiological dormancy require either a cold stratification (moist chilling) or warm stratification (warm moist conditions) before they can germinate at intermediate temperatures. Other dormancy-breaking factors include nitrogenous compounds that signal sufficient soil nutrients for subsequent plant growth and smoke that signals opportunities for rejuvenation of vegetation after fire. Thus, finding the optimal environmental conditions requires multiple germination tests and relatively large seed numbers. For conservation purposes, this is not desirable and an alternative test that requires smaller seed numbers would be appealing. Such a test ideally distinguishes dormancy from viability through positive noninterfering test scores for each of these qualities. Not only is a positive test desirable, but speed of the test is an important aspect as well. Dormancy release and completion of germination can take up to a few months, if not longer, in many wild species. A faster test would be of great benefit in those extreme cases. Creating understanding of various aspects of dormancy through fundamental research should eventually lead to a better dormancy test, useful for the agricultural industry and seed banks. Such a test would satisfy the need to accurately determine seed quality.

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Part II

Breaking Seed Dormancy and Standardizing Seed Dormancy Research

Chapter 3

Standardizing Seed Dormancy Research

Henk W.M. Hilhorst

Abstract

Seeds are very attractive and convenient for molecular genetic studies that challenge principal biological phenomena related to the initiation and suppression of growth (e.g., germination and dormancy, respectively). The number of reports in this field is rapidly expanding. Seed dormancy is a widely misinterpreted biological attribute. One of the main reasons is the general neglect of reliable dormancy assays; often, the sole criterion of current dormancy assays is the total germination of a seed population after a defined period of time. This is a very insensitive and inaccurate method, particularly when comparing dormancy levels of seeds from different genotypes, seeds subjected to different treatments, or seeds originating from different environments. Other seed parameters are far more useful. Furthermore, before undertaking comprehensive molecular and biochemical studies to elucidate the underlying mechanisms of dormancy of a given species, it is pivotal to determine the general types or categories of dormancy that are imposed and whether these are influenced by the external environment. Research strategies should be adjusted to this. In order to distinguish dead from dormant seeds, a viability test should be developed. This chapter addresses in a very general way these pitfalls in dormancy research with a focus on current plant model systems in molecular genetics, such as *Arabidopsis thaliana* and *Medicago truncatula*.

Key words: Seed dormancy, Dormancy classification, Embryo, Endosperm, Germination, Seed coat

1. Introduction

1.1. Definition and Types of Dormancy

Seed dormancy is very often regarded as the absence of germination. However, the absence of germination can have several causes: (1) the seed is nonviable; (2) the environment is nonoptimal for germination; or (3) the seed or dispersal unit is dormant. From this, a practical definition of dormancy can be formulated: "Dormancy is the absence of germination of a viable seed under conditions that are favorable to germination." The ecological relevance of seed dormancy can be defined as an effective delay of germination to

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avoid germination and subsequent growth under unfavorable climatic conditions.

For meaningful genetic and molecular studies of seed dormancy, it is essential to know the type of dormancy of the species under study. The occurrence or absence of seed germination is usually the net result of two opposing forces, namely, the "thrust" force of the embryo (embryo growth potential) and the restraints exerted by tissues surrounding the embryo. A currently accepted dormancy classification (1) is based on the seed components that inhibit germination: embryo (endogenous) and seed coat (exogenous, including endosperm, perisperm, or megagametophyte, as well as fruit tissues that are part of the dispersal unit) (Table 1). In addition, different levels of intensity have been described for each of the dormancy classes, as well as combinations of dormancy classes, such as morphophysiological dormancy and combinational dormancy (a combination of physical and physiological dormancy) (1). An example of the latter can be found in the model species *Medicago truncatula*. In order to break dormancy of this species, a sulfuric acid treatment is required to break the physical dormancy component (hard seed coat); an additional moist-chilling treatment breaks the physiological dormancy component in the embryo (Fig. 1).

An additional classification of dormancy is based on the timing of its occurrence: primary dormancy indicates the type of dormancy that occurs prior to dispersal as part of the seed's developmental program and includes all the dormancy classes and their combinations. Secondary dormancy denotes the reacquisition of dormancy in a mature hydrated seed as a result of the lack of proper conditions for germination (3). Since secondary

Table 1
A simplified classification of dormancy

Class	Physiological dormancy	Morphological dormancy	Physical dormancy
Location of block	Metabolic block(s) in embryo	Immature embryo	Maternal tissues (seed coat), including perisperm, endosperm, megagametophyte, and fruit tissues
Physiological mechanism	Arrest of growth in apical and root meristems	Incompletely matured embryo in ripe seed	Inhibition of water uptake; mechanical restraint preventing embryo expansion; modification of gas exchange; prevention of leaching of inhibitors from embryo

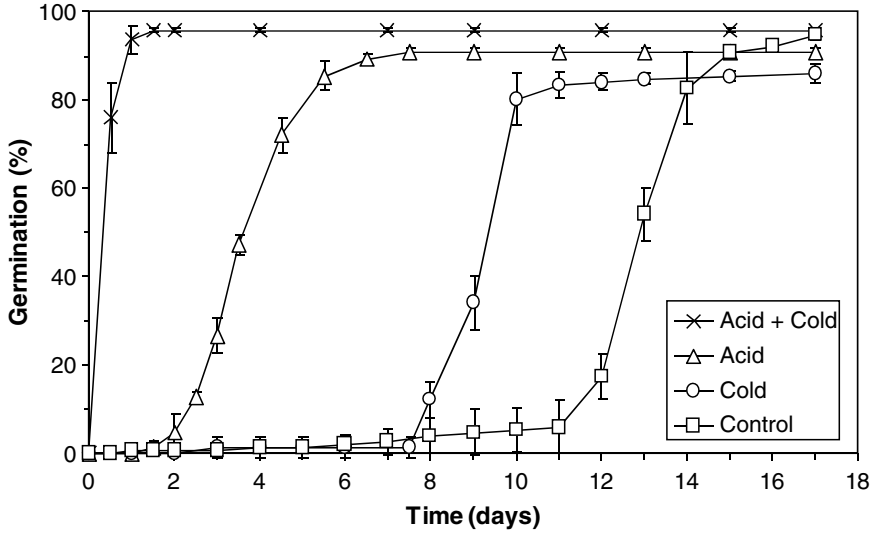

Fig. 1. Germination of *Medicago. truncatula* seeds at 20°C after various treatments for dormancy release: chemical scarification with sulfuric acid; cold imbibition (moist chilling) at 4°C for 36 h; chemical scarification plus moist chilling; and control. Each data point is the mean of three replications of 50 seeds. *Bars* represent standard deviation. Figure from Faria et al. (2) with permission from Oxford University Press.

dormancy may be repetitively broken and induced in the soil (see Chapter 5), it necessarily belongs to the class of physiological dormancy because of its reversible nature.

1.2. Seed Dormancy and the Environment of the Parent Plant

In addition to genetic factors, the environment may have a profound influence on the acquisition of dormancy during seed development. As yet, we cannot generalize as to which environmental factors result in consistent effects on dormancy over a range of species. In the literature, scattered reports can be found on a variety of environmental factors, including day length, light quality, mineral nutrition, competition, temperature, physiological age of plants, and position on the parent plant. A summary of these factors and their effects on dormancy can be found in Baskin and Baskin (4). Because of this, seed production for dormancy research is extremely demanding concerning precise control over the plant growth environment. For small-seeded species, such as *Arabidopsis*, the use of climate rooms may guarantee fairly constant environments but for larger species, such as tomato, seeds are usually produced in greenhouse environments, which are much more variable.

1.3. The Breaking of Seed Dormancy

As already exemplified above, different classes of dormancy require different methods of breaking dormancy. Under natural conditions, physical dormancy may be broken by factors such as fire, alternating high and low temperatures and humidity, or passage through an animal's digestive tract. In the lab, breaking of physical dormancy

usually requires fairly severe scarification, e.g., by mechanical means, such as abrasion or cutting, or by the controlled application of acid or boiling water. Physiological dormancy may be broken by dry after-ripening for often extended periods of time. After-ripening may be accelerated by elevating the storage temperature, but this also accelerates seed aging. A very common method to break dormancy is the exposure of imbibed seeds to cool temperatures between 2 and 10°C (cold stratification or moist chilling). In many species, including *Medicago*, *Arabidopsis*, and tomato, a few days are sufficient. However, seeds from species with a much deeper (physiological) dormancy may require several months of cold stratification to break dormancy (see Chapter 4). Seeds with morphophysiological dormancy may require a combination of moist-chilling and warm (>15°C) stratification to break physiological dormancy and promote late maturation or pregerminative (i.e., prior to radicle protrusion) growth of the embryo, respectively.

Gibberellins (GAs) are generally very effective in the breaking of dormancy. Application of GAs may often circumvent the need for "conventional" dormancy-breaking factors, such as moist chilling, light, and nitrate. However, it should be noted that seeds with deep physiological dormancy may not respond to GAs and require long periods of moist chilling (1).

1.4. The Assessment of Seed Dormancy

Although the assessment of dormancy of a batch of seeds seems straightforward, the results may lead to erroneous conclusions. It is common practice to subject seeds to a standard germination test at a certain temperature thought to be optimal for germination for a certain period of time, after which germination is counted. If germination is 100%, it may be concluded that the seeds are nondormant and at 0% germination, they are considered fully dormant. Any scores between these values are interpreted as "partially dormant." However, dormancy is very much defined in relation with the environment (5). For example, seeds with little dormancy have a much wider range of permissive temperatures for germination than seeds with deeper dormancy. Thus, seeds may appear dormant at one temperature and nondormant at another. When comparing different accessions, mutants, or cultivars of a species, a simple end-point germination test may not be sufficient to discriminate differences in dormancy (Fig. 2a). A more discriminative test assesses germination at several temperatures in order to estimate the width of the temperature window for germination. The generation of complete germination-over-time curves may be more discriminative, even at a single temperature. The more sensitive parameter to be determined is the time to 50% germination, t_{50} (Fig. 2b). In extreme cases, it can be seen that different seed batches that were originally assessed as nondormant (100% germination) are now displaying a range of dormancy levels (Fig. 2b).

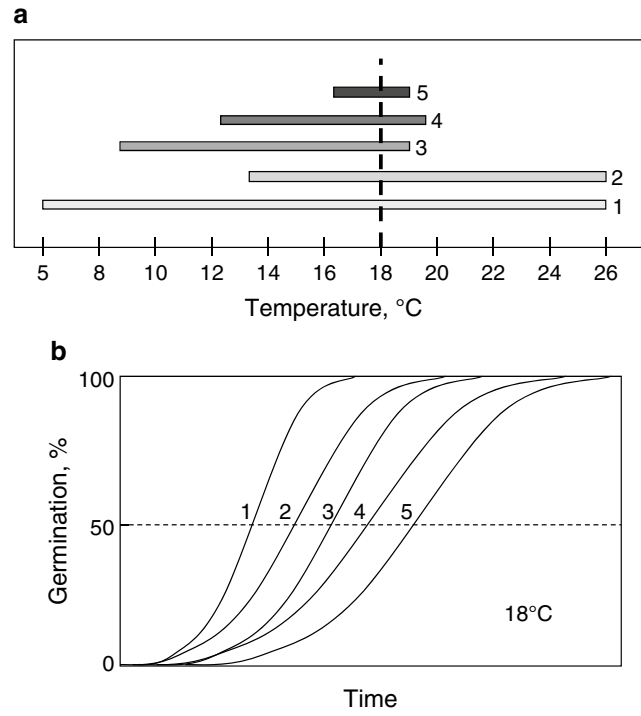

Fig. 2. A comparison of seed batches with different levels of dormancy. (**a**) Germination of five seed batches with different levels of dormancy. A wide thermal range is associated with a low dormancy level, and a narrow thermal range indicates deeper dormancy. Dormancy level increases with *gray scale* in figure. *Dotted line* indicates temperature at which all seed batches germinate, suggesting an absence of dormancy. (**b**) Germination-over-time curves of the five seed batches at 18°C, demonstrating differences in dormancy expressed in different t_{50} values, but not in final total germination.

2. Materials

2.1. Seed Production, Harvesting, and Storage

Required materials for seed production depend on the species. Here, only some general recommendations are given. For specific details, the reader is referred to the handbooks or common protocols for the species under study, e.g., *A. thaliana* (6) and *M. truncatula* (7).

1. Plant growth conditions should be as controlled as possible. This can be attained by using climate rooms for the smaller species or greenhouses for larger species. However, it is almost impossible to produce homogeneous plant populations at different points in time because even under controlled conditions, seasonal variation cannot be eliminated (see Note 1).
2. Besides the growing space, all materials, such as soil, pots, nutrition, watering, and irradiation, should be as constant as possible throughout the plant population (see Note 2).

3. Standard refrigerator-type incubator set at 20°C with a container on the bottom shelf containing a saturated solution of magnesium chloride, creating an atmosphere of 32% RH (see Note 3).
4. An oven with temperature set at 103°C for moisture content determination.

2.2. Assessment of Seed Dormancy

1. Several incubators to create a range of germination temperatures with a minimum of three, e.g., 10, 20, and 30°C. Light intensities and light regimes should be the same in each incubator. Alternatively, a thermogradient table may be used (see Note 4).
2. Gibberellic acid 4+7 (e.g., Berelex, ICI, UK).
3. Petri dishes of 50 or 90 mm in diameter or larger boxes, depending on seed size.
4. Filter paper (e.g., Whatman No. 1) with circle diameters of 50 and 90 mm, in larger sheets or in harmonica sheets of paper for large seeds.
5. Forceps, stereo microscope for small seeds.
6. Optional: Software to generate germination-over-time curves and parameters. For example, SeedCalculator (Plant Research International, Wageningen, The Netherlands).

2.3. Dissection of Seeds to Study the Contribution of Seed Components to Dormancy

1. Forceps, tweezers, scalpel, and razor blades to dissect seeds.
2. Dissection microscope.
3. Liquid nitrogen.

2.4. Determining Viability of a Seed Batch

1. Forceps, tweezers, scalpel, and razor blades to dissect seeds.
2. Dissection microscope.
3. 2,3,5-triphenyltetrazolium chloride (tetrazolium): 1% solution (v/v).

3. Methods

3.1. Seed Production, Harvesting, and Storage

1. Growing plants for seed production should be completely randomized. This means that plants within a climate room or greenhouse should be moved to another position within this space on a daily basis. Greatest spatial effects can be expected along edges of the plant population (see Note 5).
2. When comparing different genotypes, all of them should be grown in the same space at the same time to synchronize developmental periods. Yet, differences in flowering time may result

in (slightly) different periods of seed development and, hence, different levels of dormancy. Also, when seed harvesting is done from all plants of one genotype at the same time, seeds will not be equally mature. Therefore, plants/flowers should be tagged at the start of flowering (see Note 6).

3. After cleaning and initial rapid drying, e.g., under a forced stream of air, harvested seeds should be equilibrated at 32% RH for several days to reduce moisture content to approximately 5–7%, on a dry weight basis, before hermetic storage at −20°C or preferably −80°C. The drying time depends on seed size (see Note 7).
4. In order to avoid freezing damage, the seed moisture content should be low enough. Therefore, the seed moisture content must be determined. Preweighed seeds are placed in an oven at 103°C for 16 h and weighed again. The moisture content can then be calculated on a wet or dry weight basis.
5. It is essential that dormancy of the seed batches is determined at time zero (i.e., at harvest). This data serves as an important reference for comparison with results from other studies and for changes in dormancy during dry storage.

3.2. Assessment of Seed Dormancy

1. The type of dormancy can best be explored by applying a number of treatments that are known to break dormancy. For example:
   (a) Dry after-ripening, moist chilling, light, nitrate, or combinations of these factors may break *physiological dormancy*.
   (b) Whole or partial removal of seed coat may break *physical dormancy*.
   (c) A combination of (a) and (b) may break combinational dormancy.
   (d) A combination of moist-chilling and warm "stratification" (a period of warm temperature, such as 25°C, in which seeds are kept moist) may break *morphophysiological* dormancy.

   It is impossible to give more detailed information because there is a wide variation in the dormancy-breaking requirements of different species. The above described strategy generally reveals the type of dormancy. However, the more intense a certain type of dormancy, the longer the treatments or the more factors required to break dormancy. For example, physiological dormancy may be subdivided into deep, intermediate, and nondeep physiological dormancy. These three levels require 3–4 months, 2–3 months, or 1 week of moist chilling, respectively, to break dormancy (ref. 1 and see Note 8).
2. Once the type of dormancy of a certain species is known, further analyses can determine differences in the depth of dormancy

among genotypes or the effect of certain environmental conditions. For this, it is important to test germination at different temperatures and generate complete germination-over-time curves (Fig. 2). Generating such a curve for a certain species or a genotype also reveals the minimum duration of the germination experiment. The experiment is not completed until maximum germination has been attained. For most of the model species, this duration is in the order of several days to some weeks, depending on the conditions. For example, germination at low temperature is generally slower than at higher temperature.

3.3. Dissection of Seeds to Study the Contribution of Seed Components to Dormancy

1. Dissect seeds rapidly and with a minimum damage into desired compartments. This means that seeds are subjected to as little stress as possible. Flash-freeze seed tissues in liquid nitrogen immediately after dissection. Seed material may then be stored frozen (−80°C) until further use (e.g., molecular analysis). It is recommended to practice seed dissection until results are satisfactory (see Note 9). It is possible to dissect even very small seeds, such as *Arabidopsis*, into embryo and endosperm and subject tissues to genomic analysis (8).

3.4. Determining Viability of a Seed Batch

It should be realized that dormancy research can be meaningful only if a reliable viability test is included. It is trivial, but evidently very important, to discriminate between dead seeds and nongerminating dormant seeds. Oddly enough, in many molecular studies of dormancy, this issue has been neglected.

1. A first inspection may already reveal whether nongerminated seeds are viable or not. Most viable seeds should resist the gentle pressure of forceps. If the seed is soft and can be mashed easily, it is probably dead. Viable embryos are usually white and firm.
2. A frequently used viability test is the application of gibberellins to induce germination. With the application of GAs, any requirement for dormancy-breaking compounds or treatments may be circumvented. However, deeply (physiologically) dormant seeds may not always respond to GAs (4). Furthermore, seeds with hard and/or thick seed coats may not respond if GAs cannot be taken up. GA_{4+7} is approximately 1,000 times more effective than GA_3, which is the most widely used gibberellin (9). An (prolonged) incubation of seeds in 100 μM GA_{4+7} or 1,000 μM GA_3 at the optimal germination temperature should be effective. It may be necessary to scarify the seeds to facilitate the uptake of GAs.
3. A vitality stain, such as tetrazolium, may be a very reliable, semiquantitative, viability test. Overnight staining of (scarified) seeds in a 0.1–1.0% tetrazolium solution at elevated

temperature (25–30°C) is usually sufficient for optimal staining. Viable seed tissues are stained brightly red, whereas dead tissues are not stained at all. Depending on the species, staining of only parts of the embryo may be acceptable, since embryos with some dead tissues can still develop into normal seedlings (10).

4. Notes

1. Rather than attempting to standardize growth conditions for seed production to compensate for seasonal variations, a standardized dormancy assay for a certain species is much more useful when comparing results from different locations and times. It is important to monitor conditions during seed production to find possible relations between the environment and dormancy of the mature seeds.
2. Attention should be given to possible temporal and spatial variations in light, nutrition, and watering. Even with a rigid randomization schedule, these variations may have an impact on dormancy, particularly when the duration of seed development is relatively short.
3. This is a very effective and inexpensive solution as compared to the purchase of incubators that have a controlled RH. Tables for equilibrium relative humidities of saturated salt solutions at different temperatures can be found in Sun (11).
4. Thermogradient tables need constant attention during germination experiments. Particularly at higher temperatures (>25°C), seed samples may dry out. Independent monitoring of temperature by placing T-probes near the seeds on the thermal plate is essential.
5. In order to eliminate edge effects, control plants may be placed along the edges of the plant population.
6. It may suffice to record the time of first flowering of each genotype and adjust the time of seed harvest accordingly.
7. There are reports of differences in seed quality as a result of the rate of drying (12). Thus, possible effects should be determined by comparing two drying rates, e.g., at different relative humidities.
8. This further subdivision into dormancy levels is solely based on practical considerations (1).
9. No matter which method is used, dissection of seeds always imposes stress to the tissues and, hence, may influence the molecular properties of the tissues, including gene expression.

Thus, dissection methods should be aimed at reducing stress. The applied stress is not only mechanical; long dissection procedures may also lead to (partial) dehydration of the tissues. It is, therefore, important to dissect seeds under high humidity, e.g., in a glove box at 100% RH.

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Chapter 4

Eyeing Emergence: Modified Treatments for Terminating Dormancy of Conifer Seeds

J. Allan Feurtado and Allison R. Kermode

Abstract

Many seeds of coniferous species display a deep primary dormancy at maturity and require several weeks of pretreatment to produce seed populations that germinate in a vigorous and timely manner. Facilitating an efficient transition from dormancy to germination by devising improved protocols for dormancy breakage is not only important to conifer seed research, aiding in the study of the dormancy process itself, but is also of interest and applicability to commercial forest nursery operations. In the forests of British Columbia, Canada, several conifer species are well-adapted to their environment, with seeds needing to experience long durations in the moist state at cool or fluctuating temperatures. These include yellow-cedar (*Callitropsis nootkatensis*), western white pine (*Pinus monticola*), and true fir species, such as Pacific silver fir and subalpine fir (*Abies amabilis* and *A. lasiocarpa*, respectively). In this chapter, we discuss the development of new dormancy-breaking protocols for the aforementioned species that centre on the balance of several key aspects: (1) reducing the time needed to terminate dormancy in the seed population; (2) synchronicity of germination; (3) ease of use; (4) cost-effectiveness; and (5) repeatability. Where possible, any new or modified protocol should be further tested in relationship to promoting rapid seedling growth in a forest nursery greenhouse setting and after planting at natural stands. Based on the five criteria listed above, very significant improvements compared to traditional dormancy-breaking methods have been achieved for the targeted conifer species. Where tested (e.g. yellow-cedar), the modified dormancy-breaking treatments result in vigorous growth in the greenhouse and after planting at natural stands.

Key words: Seed dormancy, Conifers, Moist chilling, Traditional dormancy-breaking protocols, Modified dormancy-breaking protocols, Alcohols, Gibberellic acid, Solid matrix priming, True firs (*Abies* spp.), Yellow-cedar, Western white pine

1. Introduction

Seed dormancy, an adaptive trait that facilitates the distribution of seed through the dimensions of time and space, has been a key trait in the evolution of both angiosperm and gymnosperm species – helping plants maximize their fitness through successful

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establishment of subsequent generations (1, 2). The capacity of the imbibed seed to modulate its developmental schedule to germinate only when environment signals predict that growth and seedling establishment will be successful is a remarkable phenomenon. However, when seeds fall into human hands for use as cultivated crops, dormancy becomes an unwanted and undesirable trait since the grower can usually ensure that conditions in cultivated fields or in the greenhouse are optimal for seedling establishment and plant growth. It is not surprising, then, that domestication of a crop almost always removes or significantly reduces seed dormancy (3, 4). However, this has not been the case for tree species used in reforestation efforts. Despite the development of a more comprehensive silvicultural programme as seen in tree seed orchards and seedling nurseries, most conifer species have significant innate primary dormancy. Of course, lack of domestication in conifer species is understandable; breeding programmes are still rather juvenile, especially compared to the typical life cycles of our tree species. And few would argue that reducing dormancy to negligible levels in conifers is a much more arduous task when compared to any current domesticated crop. Fortunately, seed dormancy in species used for reforestation efforts is still a much needed trait; once the window of seed production and seedling establishment associated with the first generation in the nursery has passed, the transition from dormancy to germination of mature dispersed seeds of the next generation must then rely on signals from the "natural surroundings". Thus, retaining an inherited mechanism for seed survival is an utmost priority. As such, conifers are rather unique among crops that are cultivated – seed dormancy remains a necessary genetic trait for re-establishment of renewable natural stands. Accordingly, rather than attempting to breed out inherent dormancy mechanisms, forest nurseries require efficient dormancy-breaking treatments to ensure timely, economical production of vigorous seedlings.

Seeds of yellow-cedar (formerly known as *Chamaecyparis nootkatensis*, and now referred to as *Callitropsis nootkatensis*), true fir (*Abies*) species, and western white pine (*P. monticola*) all display deep dormancy at dispersal (5–7) and are prime examples of species that require efficient dormancy breakage in tree seed nurseries. Their deep physiological dormancy is, of course, due to the natural habitat in which each species exists.

The natural habitat of yellow-cedar occurs along the Pacific Northwest, through Oregon to British Columbia (BC), and northwards to Alaska (8, 9). Yellow-cedar has a long reproductive cycle, taking between 2 and 3 years to produce seeds (10). Problems with yellow-cedar seed production often occur because of poor reproductive success in its natural habitat. In seed orchards, which are usually established at lower elevations (11), cone production is

likewise inconsistent from year to year. Non-optimal environmental conditions associated with seed orchards (e.g. low elevation, warmer temperatures, and prevalence of insects) may further lead to problems with seed maturation, and often seeds are empty or only partially filled at maturity and the rates of seed abortion are high. We have also observed a high frequency of seeds in which there is development of the megagametophyte in the absence of the embryo. Exacerbating problems of poor seed production are problems associated with inadequate dormancy termination; as a result, this species is at a competitive disadvantage. In nature, only a low percentage of seeds germinate the first year after dispersal. The majority of seeds need longer durations (sometimes, up to another year) of moist chilling to break dormancy. Throughout this time, seed numbers can decline dramatically due to consumption by birds and small animals or deterioration caused by fungal attack (12). Currently, the common or traditional method of breaking dormancy of yellow-cedar seeds in a lab or forest nursery setting is approximately 3 months in duration, consisting of a 3-day running water soak (10–20°C), 4 weeks of warm "stratification" (in which seeds are maintained in the moist state at ~21°C), and 8 weeks of moist chilling (2–4°C) (7, 13).

Western white pine grows along the west coast in southern British Columbia and southwards through Washington, Oregon, and Northern California. In the interior of North America, western white pine can be found in pockets of Idaho and Montana (14). Western white pine was a very important commercial species in the nineteenth century; however, due to the North American introduction of white pine blister rust from infected pines in Europe and the subsequent death of a great proportion of the trees, its commercial impact is limited even today. However, rust-resistant stocks of western white pine are being produced and the impact of the disease today has mostly been mitigated (15, 16). In order for germination to be elicited, seeds must be subjected to a prolonged moist-chilling treatment for 3 months or longer (17). The standard method used in British Columbia (e.g. at the BC Ministry of Forests and Range, Tree Seed Centre, Surrey, BC) is a 14-day running water soak (10–15°C) and 98 days of moist chilling at 2°C (13). Yet, even with this lengthy moist-chilling treatment, the germination capacity of different seedlots can be vastly different. Differences in germination performance can also occur when the same seedlot is chilled at different times or when there are large numbers of seeds subjected to the treatment (e.g. in a commercial operational setting) (17, 18).

Pacific silver fir (*A. amabilis*) trees grow throughout Oregon, Washington, and north through coastal British Columbia into southern Alaska. Subalpine fir (*A. lasiocarpa*) has one of the widest distributions of any fir; its habitat occurs from New Mexico, Arizona, and Northern California into Oregon, Washington, and

north into British Columbia and Alberta, stopping in southern Alaska and the Yukon (19, 20). Seeds of true fir species, including Pacific silver fir and subalpine fir, display pronounced seed dormancy at maturity. Accordingly, breaking dormancy in these species requires a prolonged moist-chilling treatment of 3–4 months (21–23). In addition, germination of these firs can be impaired by seed pathogens and select seedlots can have high numbers of empty seeds (24). Seeds of the true firs can be prone to germination during moist chilling; thus, a re-drying of the seeds during chilling has been incorporated to prevent this (21). A typical protocol for dormancy termination of Pacific silver and subalpine fir seeds consists of soaking of seeds for 2 days, followed by moist chilling (2–4°C) at >45% moisture content for 4 weeks. Subsequently, seeds are subjected to a "dry-back" (to 30–35% moisture), which is employed to inhibit germination and control fungal growth during moist chilling. Following "dry-back", the seeds are moist-chilled for an additional 8 weeks. Thus, the total protocol is approximately 3 months in duration (13).

Below, we review and summarize modified dormancy-breaking protocols for yellow-cedar, western white pine, true firs, Pacific silver fir, and subalpine fir, previously published as separate entities (Fig. 1) (5, 6, 25). In brief, after testing various methods to

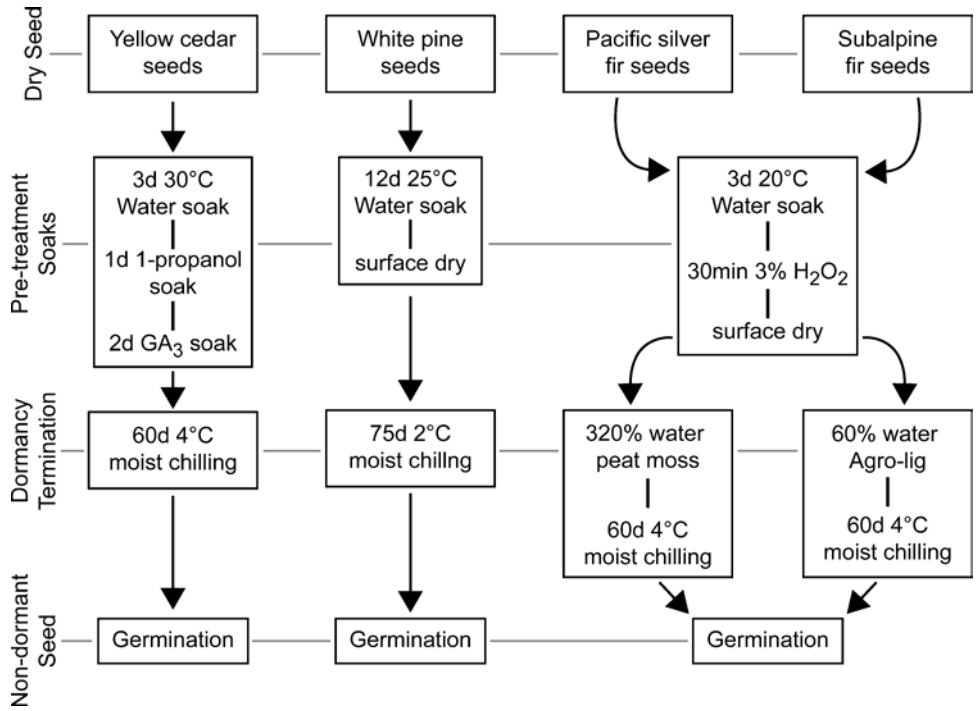

Fig. 1. Flow chart summarizing the modified dormancy-breaking methods for yellow-cedar, western white pine, and Pacific silver and subalpine firs.

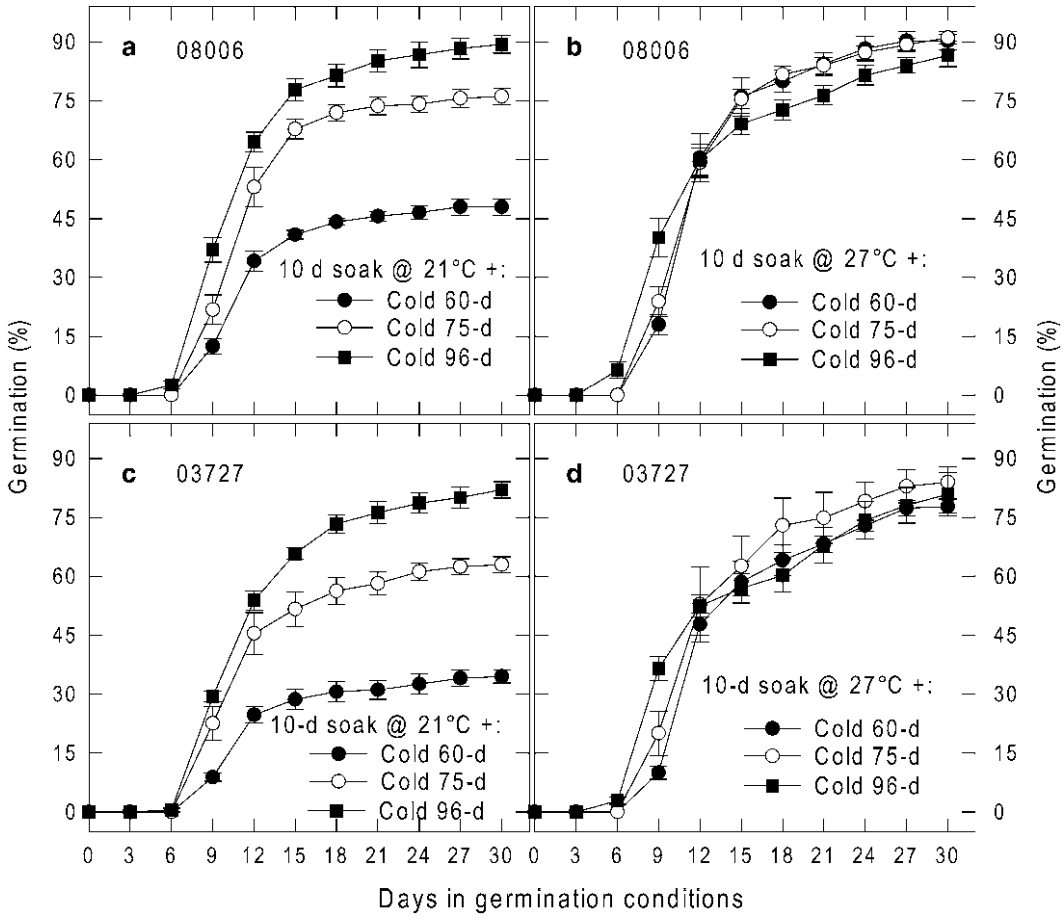

Fig. 2. Germination of western white pine seedlots 08006 (**a**, **b**) and 03727 (**c**, **d**) following a water soak and moist chilling. Seeds were either soaked at 21°C (**a**, **c**) or 27°C (**b**, **d**) for 10 days, followed by 60, 75, or 96 days of moist chilling. Reprinted from (6) with kind permission from the International Seed Testing Association.

effectively break the dormancy of yellow-cedar seeds (25–29), the most effective uses the anaesthetic 1-propanol, combined with a 3-day warm water soak, a 2-day treatment with the hormone gibberellic acid, followed by 30–60 days of moist chilling. For western white pine, a prolonged warm water soak followed by a 75-day moist-chilling period is most proficient (Fig. 2) (6). Finally, moist chilling within a solid matrix of Agro-Lig (a commercial formulation of humic acids) or peat moss proves very effective for breaking dormancy of Pacific silver and subalpine fir seeds, respectively (Table 1) (5). Compared to traditional dormancy-breaking methods, the modified protocols significantly reduce the time needed to terminate dormancy. The methods are effective for various seedlots within each species, and they further lead to synchronous germination within the seed population. For yellow-cedar, the modified protocols proved to be further effective in

Table 1
Germination percentages of fir seeds after different SMP-chilling treatments. Modified from (5) with kind permission from Springer Science and Business Media

		Moist chilling (weeks)		
Fir species	**Treatment**	**4**	**8**	**12**
Pacific silver fir	Control: moist chilling	39	44	45
	Agro-Lig Greens Grade	47	64	44
	Sand	61	65	57
	Peat moss	62	**79**	56
	Soaking only	3	3	3
Subalpine fir	Control: moist chilling	41	58	63
	Agro-Lig Greens Grade	63	**83**	70
	Sand	51	71	62
	Peat moss	55	71	68
	Soaking only	12	12	12

Bold numbers represent the most efficient dormancy-terminating protocol.

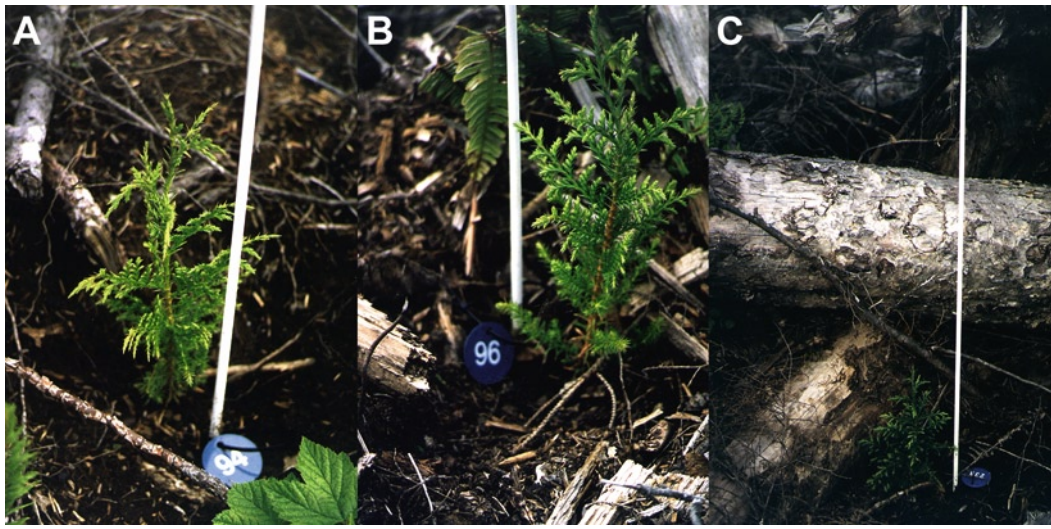

Fig. 3. Growth of yellow-cedar seedlings 2 months after out-planting into natural stands near Port McNeill, BC, Canada. These seedlings were subjected to the dormancy-breaking protocol described for yellow-cedar (Subheading 3.1). Reprinted from (29) with kind permission from Springer Science and Business Media.

promoting vigorous growth in a forest nursery greenhouse setting and after planting at natural stands (Fig. 3). These protocols described below in detail are the collaboration of several years of work in the laboratory of A. Kermode.

2. Materials

2.1. General Items

1. Polystyrene square container 156C, 10.95 mm×10.95 cm× 3.5 cm (Hoffman Manufacturing, Jefferson, OR, USA).
2. Seedburo K-22 seed germination paper (Kimpak), No. 87, 25.4×35.6 cm (Seedburo Equipment, IL, USA), cut to fit inside the container described in item 1 above.
3. Whatman 3MM Chromatography Paper, 35×45 cm, cut to fit inside the container described in item 1 above.
4. Forceps (Fine Science Tools, North Vancouver, BC, Canada).
5. Analytical scale (for milligram measurements).
6. Laminar flow hood for sterile work and air drying of seed (ENVIRCO, Sanford, NC, USA).
7. Spoonula or Spatula.
8. Mesh bag made from vinyl-coated fibreglass screen material available from most building supply stores.
9. Stainless steel tea ball available from most grocery stores.
10. Double deionized sterile water (dH_2O) (double deionized H_2O, ddH_2O, is sterilized by autoclaving).
11. Water bath (approximately 10–12 L) (e.g. Thermo Scientific, Newington, NH, USA).
12. Refrigerator capable of maintaining 2–4°C.
13. Controlled temperature growth chamber (e.g. Conviron TC16, Controlled Environments Ltd, Winnipeg, MB, Canada).

2.2. Dormancy Termination of Yellow-Cedar Seeds

1. 1-Propanol (Anachemia Science, Montreal, Canada).
2. Gibberellic acid A_3 (GA_3) (Sigma-Aldrich, Oakville, ON, Canada).
3. Petri dishes (10×1.5 cm).
4. Parafilm.
5. Mature seeds of yellow-cedar. In our case, we obtained all seedlots from the BC Ministry of Forests and Range, Tree Seed Centre (Surrey, BC, Canada). Seeds should be maintained at –20°C before use.

2.3. Dormancy Termination of Western White Pine Seeds

1. Petri dishes 10×2.5 cm.
2. Optional: 70% ethanol, 30% sterile water.
3. Optional: 10% commercial bleach, 90% sterile water.
4. Optional: Corning 150-mL Tube Top Vacuum Filter System (Fisher Scientific, Ottawa, Canada).

5. Mature seeds of western white pine obtained from the BC Ministry of Forests and Range, Tree Seed Centre (Surrey, BC, Canada). Seeds should be maintained at –20°C before use.

2.4. Dormancy Termination of Pacific Silver Fir and Subalpine Fir Seeds

1. 30% hydrogen peroxide (H_2O_2).
2. Agro-Lig Greens Grade (humic acids with particle sizes between 0.212 and 1.29 mm) (America Colloid Company, Reeder, ND, USA).
3. Peat moss (Lakeland Peat Moss Ltd., Edmonton, AB, Canada) sieved with 1.4-mm testing sieve (see item 6 below).
4. 150-mL sterile sample bags (Fisher Scientific).
5. Testing sieve #14, 1.4-mm screen opening (VWR Canlab).
6. Mature seeds of Pacific silver and subalpine firs obtained from the BC Ministry of Forests and Range, Tree Seed Centre (Surrey, BC, Canada). Seeds should be maintained at –20°C before use.

3. Methods

3.1. Dormancy Termination of Yellow-Cedar Seeds

1. Following removal of the seeds from storage at –20°C, seeds, in batches of 150, are soaked within tea strainers or mesh bags in a 30°C water bath for 3 days.
2. Seeds are then transferred to Petri dishes containing 30 mL of 50 mM 1-propanol. The Petri dishes are sealed with parafilm and gently agitated at 70 rpm on a platform shaker for 24 h.
3. Seeds are transferred to a Petri dish containing 30 mL of 200 mg/L of GA_3. Plates are sealed and agitated as in step 2 for 48 h.
4. The seeds are then placed directly on a moist substratum within a polystyrene container for 60 days at 4°C in the dark. The moist substratum consists of one layer of Whatman 3MM paper and one layer of Kimpak in a square polystyrene container ($10.95 \times 10.95 \times 3.5$ cm) moistened with 30 mL of sterile ddH_2O.
5. Following moist chilling, seeds are divided into replicates of 50 seeds each and placed on a moist substratum in polystyrene containers for germination. This moist substratum is identical to that described in step 4, except 50 mL of ddH_2O is added.
6. Seed containers are placed in germination conditions: 30°C days, 20°C nights with an 8-h photoperiod; light intensity at 25 μmoles/m^2/s, PAR 400–700 nm.
7. Germination counts can be monitored daily for the first 15 days followed by once every 3 days for 15 days (30 total days).

3.2. Dormancy Termination of Western White Pine Seeds

1. Following storage of seeds at –20°C, western white pine seeds are allowed 1–2 h to equilibrate to room temperature. During this time, seeds can be counted into needed replicates for subsequent experiments. Alternatively, if dealing with large numbers of seeds, one can calculate the number of seeds per gram and weigh the seeds.
2. Optional: Depending upon the seedlot and the presence of seed-borne pathogens, seeds may need to be sterilized. Seeds can be sterilized by soaking in 70% ethanol for 5 min, followed by 10% commercial bleach for 3 min. This is followed by rinsing with sterile water for two 5-min periods. We often use bottle-top filters to ease in the sterilization of seeds (e.g. Corning 150-mL Tube Top Filter).
3. Seeds are placed into water-penetrable containers, such as stainless steel tea balls or, if using larger quantities of seeds, into screen or mesh bags.
4. Seeds are soaked for 10–14 days in running tap water (approximately 10–15°C) or in a 25°C water bath with water exchanged daily (see Note 1). If you are using aseptic technique, the seeds can be soaked in 50-mL conical tubes or Erlenmeyer flasks with gentle shaking.
5. Following the water soak, water is drained and the seeds are placed on Whatman 3MM paper in a flow hood to dry the surface moisture off of the seeds (see Note 2).
6. Seeds (<250 per container) are placed on Whatman 3MM paper supported by K-22 germination paper in a square polystyrene container to which 25 mL of ddH_2O is added. If you are using aseptic technique or have small sample sizes (e.g. 25–30 seeds per replicate), deep dish Petri plates (10×2.5 cm) are used and 12.5 mL of ddH_2O is added.
7. Seeds are placed to moist chill at 2–4°C for 98 days in the dark (see Note 3).
8. Following moist chilling, the seeds can be transferred to germination conditions. The seeds are transferred to new square polystyrene containers (50 seeds per dish) or deep dish Petri plates (25 seeds per plate), set up as mentioned in step 6, except with 50 and 23 mL of ddH_2O, respectively. Germination conditions of 23°C and a 16-h photoperiod or 25°C days, 15°C nights, and an 8-h photoperiod have been used successfully (see Note 4). Light intensity for both conditions is kept at approximately 60–80 μmol/m²/s, PAR 400–700 nm.
9. Germination counts can be monitored daily for the first 15 days followed by once every 3 days for 15 days (30 days total).

3.3. Dormancy Termination of Pacific Silver and Subalpine Fir Seeds

1. Following removal of the seeds from storage at −20°C, seeds are weighed to estimate the seed number needed for a typical experiment.
2. Seeds are soaked in a mesh bag submersed in an aerated-dH_2O running water bath for 3 days at 20–22°C (see Note 5).
3. Seeds are sterilized for 30 min in 3% H_2O_2 and rinsed several times with sterilized ddH_2O (see Note 6).
4. Seeds are dried for approximately 5 min in a laminar flow hood until the seed-surface moisture disappears. During this time, seeds are divided into the replicates for the experiment (e.g. 4 replicates of 50 seeds each).
5. During the final hours of the seed soak, the matrices for solid matrix priming (SMP) chilling are prepared and placed in 150-mL sample bags. For Pacific silver fir seeds, pre-sieved peat moss is combined with 320% sterile ddH_2O on a weight-to-weight (w/w) basis (see Note 7) and approximately 30 mL of wetted matrix is placed into a 150-mL sample bag together with 50 seeds from step 4. For subalpine fir seeds, Agro-Lig Greens grade is mixed with 60% sterile ddH_2O on a w/w basis and approximately 30 mL of wetted matrix is placed into a 150-mL sample bag together with 50 seeds from step 4.
6. Seeds within the various matrices are then placed at 4°C for 8 weeks of subsequent moist chilling (see Note 8).
7. Following 8 weeks of SMP chilling, fir seeds are removed from the matrices by rinsing in a sieve (1.4-mm screen) with dH_2O water.
8. Approximately 50 seeds are then transferred to a square polystyrene container with Whatman 3MM paper supported by K-22 germination paper and 50 mL of dH_2O water is added.
9. Germination is then tested using 21°C days, 15°C nights with an 8-h photoperiod; light intensity is at 40 μmoles/m^2/s, PAR 400–700 nm.
10. Germination can be monitored daily or every 3 days for a total period of 30 days. To estimate the "realistic germinability" of a particular seedlot, the ungerminated seeds are then cut open to determine the proportion of unfilled seeds (e.g. seeds missing a viable embryo due to abortion or poor seed developmental conditions, or attached by a parasite or a microorganism, such as fungus).

4. Notes

1. Using a higher temperature soak (i.e. 25°C) decreases the subsequent moist-chilling duration (6).
2. This step helps minimize fungal contamination and also aids in dormancy breakage possibly due to increased air exchange (5, 6).

3. The moist-chilling time for western white pine seeds can be shortened to 75 days if a higher temperature soak is used (Subheading 3.2, step 4).
4. Germination conditions at 23°C constant temperature elicit more effective germination (i.e. germination occurs over a shorter time frame), but 25°C days, 15°C nights may more accurately reflect conditions in a forest seed nursery.
5. In our experience, a 3-day soak is necessary to obtain a stable water content (e.g. 45% for Pacific silver fir seeds and 50% for subalpine fir seeds).
6. Sterilized water may not be needed at this point and for subsequent moist chilling since most of the fungal contamination, if present, comes from the seeds themselves.
7. Moisture content of the matrices is calculated on a weight-to-weight basis and is calculated based on: weight of water/dry weight of solid matrix × 100.
8. To determine the optimal period of moist chilling, we also considered the potential for germination during the chilling period and tried to minimize this value. Thus, longer periods of SMP moist chilling are beneficial to dormancy breakage and subsequent germination, but unwanted "pre-" germination was always increased – sometimes as much as 20%.

Acknowledgements

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Chapter 5

Production of Seed Samples for the Effective Molecular Analysis of Dormancy Cycling in *Arabidopsis*

Steven Footitt and William E. Finch-Savage

Abstract

Most often, the samples used for molecular analysis of dormancy are populations of seeds. An essential survival characteristic of seed populations inhabiting the variable surface layers of the soil is that individuals in the population do not behave uniformly. In addition, seed dormancy (SD) status of the whole population constantly changes even in the dry state. For these and other reasons, production of appropriate and adequately characterized seed samples is the key to the correct and most informative interpretation of molecular studies. This is particularly important when the aim is to describe and explain seed behaviour in the natural environment. Molecular studies of seed dormancy, and especially ecologically relevant behaviour, such as dormancy cycling, should therefore involve characterization of dormancy status based on a sound understanding of seed physiology. This chapter discusses the problems and pitfalls of using *Arabidopsis* and provides protocols devised for use with the *Arabidopsis* ecotype Cape Verde Islands for the production and characterization of samples to be used in molecular analysis of dormancy transitions and cycling.

Key words: *Arabidopsis*, Germination, Dormancy cycling, After-ripening, Ecology

1. Introduction

Dormancy is an innate seed characteristic that defines the conditions required for germination, and therefore any signal that widens the conditions under which germination is possible should be regarded as a dormancy release factor (1). A range of these factors can act singly or in combination to release or induce dormancy. Species and different accessions of those species are peculiarly adapted to their original climate and consequently differ dramatically in their response. The choice of species and accession should, therefore, be appropriate for the experiments to be carried out.

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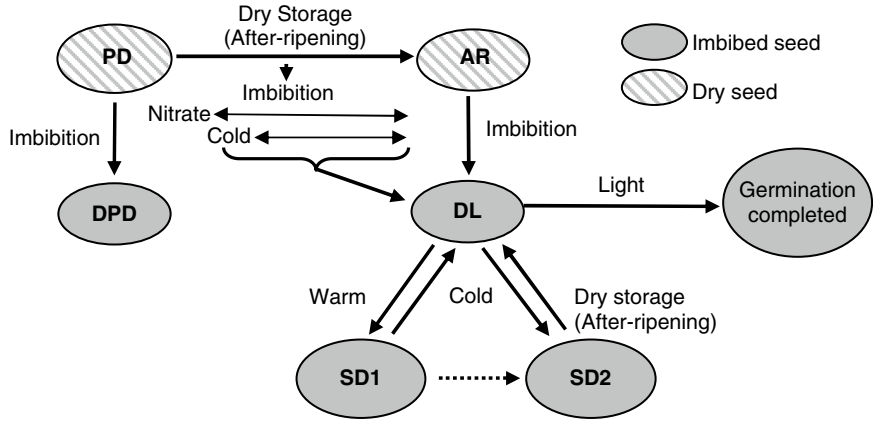

Fig. 1. Dormancy cycling in the laboratory. *Arabidopsis* (Cvi) seeds are shed with non-deep physiological dormancy, which is imposed by the tissues surrounding the embryo (the endosperm and testa). A range of environmental signals relieve this dormancy, including dry storage (after-ripening, AR), temperature (cold), light, and nitrate. However, these signals must be experienced in the correct order to break dormancy. The newly mature primary dormant (PD) seed is initially not sensitive to dormancy-relieving treatments, but can be induced to deeper dormancy (DPD). PD seeds first require some AR to develop sensitivity to nitrate and later to cold. Sensitivity to light is acquired after prolonged AR, AR followed by cold, or AR followed by nitrate. In this dormant, light-requiring (DL) state, light is required to terminate dormancy and initiate the completion of germination. If the imbibed DL seed is not exposed to light, it exhibits secondary dormancy (SD1). Secondary dormancy can be relieved by cold, but will return if seeds are not exposed to light. Longer exposure to either of these conditions deepens secondary dormancy (SD2). At this stage, after-ripening is required to relieve dormancy. Figure modified from (4) with permission from Elsevier Ltd.

Figure 1 describes the impact and interaction of factors that are relevant to the *Arabidopsis* accession Cape Verde Islands (Cvi), which is becoming the preferred model for dormancy studies. In other commonly used *Arabidopsis* accessions, the impact and interaction of these factors can be shallow and less well-defined making them less suitable for analysis. For example, a particular feature of seeds of the ecotype Cvi is that they have an absolute requirement in the whole population for exposure to light before the germination process can progress to completion. Light is, therefore, a well-defined signal that removes the final "layer" of dormancy (2, 3). For sample collection, this means that it provides a way to separate molecular changes associated with transitions between dormant states and downstream changes associated with the process of germination and radicle emergence (germination completion).

Freshly harvested (shed) seeds of Cvi have primary dormancy (PD), which has been induced during seed maturation in the mother plant. Subsequent dormancy release under natural conditions in the field may involve the same factors that are commonly used in laboratory experiments (Fig. 1). These factors are either after-ripening (AR) in the relatively dry state or a range of factors in the imbibed state. Once primary dormancy has been relieved in response to prevailing environmental conditions, secondary dormancy (SD) soon starts to be induced if the conditions required to

terminate dormancy and induce germination are absent (i.e. light). These transitions are associated with annual dormancy cycles in the seed bank. Secondary dormancy can be lost and reintroduced repeatedly as seasons change until the required germination conditions become available or until viability is lost. These changes in the depth of dormancy during transitions are gradual. Seeds in the intermediate states are said to have conditional or relative dormancy because the range of environmental factors that are permissive to germination is limited. The degree of limitation changes with the depth of dormancy. Thus, as dormancy is lost, the germination window (permissive range of environments) progressively opens and then closes as dormancy is induced into a deeper state. As samples are populations of seeds, changes are occurring at different rates in all the seeds within the population. Changes in dormancy status occur throughout seed production and at anytime afterwards at a rate determined by the environment. The dormancy status is, therefore, never fixed unless imbibed seeds are frozen to a level at which the seeds are dead upon imbibition.

Ideally, all seeds should be in the same state, and thus PD seeds should be sampled immediately after harvest as they have already begun to after-ripen. The rate of after-ripening is directly related to the level of hydration (water potential) and temperature (1, 5). Therefore, if dry seeds are to be kept for any length of time, they should be equilibrated to a low relative humidity and stored at −80°C to minimize changes. We have found that changes appear to continue during after-ripening that influence gene expression upon imbibition, even when the seeds are fully germinable (upon imbibition) and dormancy appears to have been relieved (DL). This should be borne in mind when designing experiments, and it may also be necessary to store DL samples under similar conditions of low hydration at −80°C. It is important to consider that any seed storage environment is also providing the conditions that drive after-ripening. This is particularly important when the same dormancy state is studied in more than one seed production run or when all treatments cannot be performed at the same time.

When dry seeds are imbibed, it is essential to imbibe all developmental stages under the same conditions and for the same period of time. For Cvi, 24 h is a convenient time at which seeds are in the lag phase of imbibition, but non-dormant seeds have not begun downstream germination processes. Further imbibition of dormant seeds may result in either relief or induction of deeper dormancy depending on the prevailing environment. For example, it is not until the third day of imbibition that PD Cvi seeds begin to synthesize ABA (6) and seeds imbibed for 30 days enter into a maintained dormant state that is characterized by gene expression that is different from that exhibited by seeds after 24 and 48 h of imbibition but is similar to that exhibited by seeds that are SD (3).

The initial seed dormancy status is very dependent on different environmental factors of the seed production environment, including temperature, the level and spectral quality of light (7, 8), and nitrate availability to the mother plant (9). For example, these factors can have a large impact on the AR requirement for dormancy loss in Cvi seeds (2). When comparisons are made between seeds from different production runs, it is important that these conditions remain consistent.

When the above considerations are taken into account and care is paid to the characterization of the dormant states, then populations of seeds can be produced in which most, if not all, seeds are in the same state. However, if temporal patterns are important and intermediate samples are collected, then this will never occur. These intermediate samples are mixed populations with varying levels of both dormancy (either PD and/or SD) and germination potential (DL). In a population with increasing germination potential, the speed of germination also changes with time. However, this problem can be minimized if seeds are collected at equivalent developmental stages or at consistent proportions of the time required to achieve the transition. The level of dormancy and germination potential of each sample must also be evaluated at the time of collection in order to place transcriptome changes in a physiological and ecological context.

2. Materials

2.1. Laboratory Materials

1. Dark room and green safe light.
2. Boxes for germination, e.g. polystyrene boxes 124 × 88 × 22 mm (L × W × H), Stewart Plastics Ltd., UK.
3. Roll of 3 MM chromatography paper, 7.5 cm wide (Camlab, UK).
4. Sealable freezer bags (20 × 28 cm and 19 × 19 cm).
5. GA_{4+7} stock solution (500 µM). Dissolve GA_{4+7} in 200–400 µl of 1 M KOH and make to an appropriate volume in 1.7 mM citric acid/3.3 mM K_2HPO_4 buffer pH 5.0.
6. GA_{4+7} 100 µM/fluridone 50 µM (Dow Chemical Co., UK.) solution. Fluridone is dissolved in 1 mL of dimethyl sulphoxide and combined with GA_{4+7} in citric acid/phosphate buffer as above.
7. Potassium nitrate, 10 mM.
8. Saturated solution of calcium dinitrate ($Ca[NO_3]_2 \cdot 4H_2O$) giving a relative humidity of 55.5% at 20°C.
9. Saturated solution of Lithium Chloride (LiCl) giving a relative humidity of approximately 15% at 5°C.

10. Ultipette™ BARKY CP-100 tips (Barkey Instruments International, UK) (see Note 1).
11. Pastettes (3 mL) (Jencons Ltd., UK).
12. Gilson pipettes.

2.2. Plant Growth Materials

1. *Arabidopsis thaliana* ecotype Cvi (accession number N8580).
2. Intercept systemic insecticide (Monro South, UK) (see Note 2).
3. Low-nitrogen potting compost, vermiculite, and sand at a ratio of 6:1:1 (see Note 3).
4. Multi-cell trays with 24 cells (cell volume 100 mL).
5. Seed trays lined with capillary matting and transparent seed tray covers.
6. Laboratory test sieve, 500-μm aperture (Endecotts Ltd., London, UK).
7. Aracons (Betatech bvba, Belgium).
8. Pollination bags (Perforated bread bags SM570Y, Sealed Air Corp.).

2.3. Field Materials

1. Metalaxyl fungicide (see Note 4) (Hockley International, UK)
2. Nylon mesh (mesh size 125 μm) (Clarcor UK, UK) (see Note 5).
3. Polyester thread.
4. Ballotini balls: 0.15–0.25-mm diameter (Potters Ballotini Ltd., UK).
5. Bag clips WeLoc® sizes PA150, PA110, and PA70 (WeLoc – Weland M. AB, Sweden).
6. Tray labels for attaching by wire to bag clips (e.g. size SL140 various colours) (BHGS Ltd., UK).
7. PVC-coated 0.6-mm gauge wire (Farnell, UK).
8. Blackout box for removing seeds from the soil in darkness. Box dimensions H × W × L, 300 × 350 × 500 m; top of the box to have two circular arm holes, 120 mm in diameter, located 1/3 of the distance along the length and 50 mm from the edge. Attach long reach gauntlets (Marigold Emperor ME106, Buck and Hickman, Coventry, UK) with retaining rings.
9. Lightproof laminated foil bags, 11 × 24 cm (Moore and Buckle, St. Helens, UK).
10. Bamboo stakes marked at one end with the chosen burial depth.
11. Temperature- and humidity-monitoring equipment, e.g. thermistor temperature probes (Betatherm, Ireland) and soil moisture sensors (SM200, Delta-T Devices Ltd., UK) linked to a data logger (Delta-T Devices Ltd., UK) (see Note 6).

3. Methods

The integration of laboratory and field-based techniques for the detailed molecular/physiological analysis of seed behaviour in an ecological setting is an expanding area as it becomes important to understand the impact of climate change on natural populations. The dynamic nature of seed dormancy means that such integration is not without its pitfalls (10); however, these protocols present a standardized approach that can be easily adapted to the species or ecotype under study.

3.1. General Aspects of Seed Production

1. For uniformity and reproducible levels of primary dormancy, plants should be raised in a growth room or a cabinet (see Note 7).
2. Adaptation of the ecotype under study to the climate of its site of origin may strongly influence the development of dormancy in the mother plant. An ecotype's origin should be considered before deciding on growth conditions. The depth of primary dormancy is strongly influenced by the available nitrate and temperature experienced by the mother plant. To produce seeds with a deeper level of primary dormancy, use a low-nitrogen compost and once plants have bolted, mature them at cool temperatures (e.g. 16 h/8 h light/dark cycle at 18°C/10°C) (8).
3. If it is intended to monitor dormancy cycling in the field using one ecotype, more than one independent biological sample is recommended (see Note 8). To this end, cohorts of plants should be grown separated from each other by distance, not time. If dormancy cycling between ecotypes or mutants is to be studied, then plants should be quarantined from each other using Aracons or pollination bags to prevent cross-pollination.

3.2. Seed Production and Harvest

1. Fill multi-cell trays with compost and place into seed trays lined with capillary matting, and then water.
2. Place some seeds on wet filter paper and transfer one or two seeds directly onto the surface of the compost using the tip of a pair of forceps. Label the tray with a tray label and cover with a seed tray cover with the vents closed (see Notes 9 and 10).
3. When seeds have germinated, open vents on covers to reduce humidity and remove covers 7 days after germination.
4. Reduce the number of seedlings in each cell to one.
5. As plants start to bolt, stake each plant to keep it upright or cover with Aracons (see earlier comments in Subheading 3.1).
6. At this stage, do not allow trays to dry out (see Note 11).
7. Once flowering has stopped, allow siliques to continue maturing until 75% of siliques appear dry and then stop watering and allow to dry (see Note 12).

8. Harvest seeds from each biological replicate separately by gently hand threshing the plants in a large paper bag or onto a large sheet of paper.
9. Clean seeds in each biological replicate by passing through a 500-μm sieve, then place seeds in paper envelopes, and equilibrate them at 15% RH/5°C over a saturated solution of LiCl for 7 days to reduce moisture content to approximately 5–7% on a dry weight basis, a point where the rate of dry after-ripening is reduced (5).
10. Store working seed lots at –20°C and bulk seed lots at –80°C to further reduce the rate of dry after-ripening.
11. Once seeds have been produced, it is essential that the dormancy characteristics of these seeds at time zero are determined (see Subheading 3.4).

3.3. Standard Germination Test

This configuration is ideal for the comparison of up to four independent biological replicates, ecotypes, and/or mutants.

1. Take two sheets of 3MM chromatography paper, mark one piece on the top left-hand corner with pencil, and divide from top to bottom into equal sectors for each population. Place chromatography paper into polystyrene boxes (124×88×22 mm) and add 8 mL of the appropriate solution.
2. Surface-sterilize seeds in a 0.125% sodium hypochlorite solution [household bleach (5% sodium hypochlorite) diluted to 2.5%] for 5 min and then wash them three times in water (see Note 13).
3. Using an Ultipette™ tip, plate out 50 seeds per sector in triplicate plates for each treatment.
4. Put plates in sealable freezer bags and incubate at 20°C under continuous light and score germination at 2–3-day intervals for up to 28 days, depending on the ecotype being used (see Notes 14 and 15).

3.4. Evaluating Changes in the Depth of Dormancy in Seeds Prior to Their Attaining the Potential to Complete Germination on Water

Physiological measures of changes in seed dormancy are required to conduct meaningful molecular analysis of dormancy cycling. The following measures of changes in the depth of dormancy and germination potential have the advantage that they can be related to both changes in gene expression and environmental conditions in the field.

3.4.1. Seed Viability

1. The viability of each sample at each harvest must be determined using 100 μM gibberellic acid (GA)/50 μM fluridone in citrate/phosphate buffer (pH 5.0) incubated at 20°C (see Note 16). If seeds do not germinate, then they are likely nonviable. However, in case of exceptionally deep dormancy, seeds may not respond to this robust treatment.

3.4.2. Gibberellic Acid Sensitivity Test (see Note 17)

1. Make up a GA_{4+7} stock solution (see Subheading 2.1, item 6) (11) and prepare a suitable dilution series in buffer (pH 5.0).
2. From the response curves generated, the GA dose for the half-maximal response ([GA]50) can be determined (11). In addition, by linear regression of the rate of germination to 50% for each concentration, the minimum [GA] yielding 50% germination can be determined.

3.4.3. Temperature Limits for Germination (see Note 18)

1. Incubate seeds at a range of temperatures (e.g. 5, 10, 15, 20, and 25°C) to monitor the decay of dormancy in the field. The addition of 10 mM potassium nitrate also gives an indication of changes in nitrate sensitivity in the field (see Note 19).

3.4.4. After-Ripening Time

By monitoring after-ripening time in seeds recovered from the field, changes in the depth of dormancy can be monitored.

1. Equilibrate seeds at 20°C and 55% relative humidity over a saturated calcium dinitrate solution (see Subheading 2.1, item 9) for 7–14 days, seal seeds in an Eppendorf tube, and store at 20°C in the dark. At regular intervals, perform germination tests on water at 20°C as described in Subheading 3.3. As seeds have already been surface-sterilized, do repeat this step.
2. From the resulting after-ripening curves for each harvest, determine the after-ripening time required for 50% of seeds to complete germination. This shows the change in dormancy status experienced in the field.

3.5. Laboratory Production of Seeds in Defined States of Dormancy Cycling

Samples during dormancy cycling illustrated in Fig. 1 (PD, DPD, AR, DL, SD1, and SD2), are produced in the following ways (see Notes 20 and 21):

1. PD seeds are sampled immediately after cleaning. Dry seeds should be extracted immediately to avoid after-ripening. Imbibed seeds, like those of any of the samples, should be frozen immediately in liquid nitrogen and stored at –80°C.
2. DPD seeds are produced by imbibing seeds in polystyrene boxes as above at 20°C and maintaining them on absorbent paper in the dark. Previous work shows that 30 days result in seeds that have a different gene transcriptional profile from seeds imbibed for 24 h, but it is likely this will happen in a shorter time.
3. AR seeds are produced by dry after-ripening PD seeds immediately after harvesting. An effective regime for AR is to equilibrate seeds over a saturated calcium dinitrate solution (55% RH) at 20°C for up to 14 days and then store in the dark at 20°C in a sealed waterproof vessel. The rate of AR can be changed by altering the temperature and equilibrium RH.

4. Unless seed production is closely controlled, it cannot be guaranteed that seeds will be fully after-ripened after a set period of time. Germination percentages on water at 20°C in the light should, therefore, be checked at regular intervals until germination of all viable seeds is complete and rapid. This can be assessed by comparing to germination on a mixture of GA and fluridone (see Subheading 3.4.1), which stimulates all viable seeds to germinate rapidly. At this point, AR samples can be taken and handled as PD samples.
5. DL seeds are produced by imbibing AR seeds at 20°C in the dark for 24 h. Prolonged imbibition under these conditions results in SD1 seeds after approximately 24 days (seeds do not germinate without 10 mM KNO_3 present); a further period results in SD2 seeds at approximately 80 days (seeds do not germinate in the presence of 10 mM KNO_3).
6. DL seeds can be derived from SD1 seeds by transferring them to 5°C for 2 days in the dark, where a proportion of the population regains the ability to germinate in the light without 10 mM KNO_3.
7. Seeds that are left at 5°C in the dark for 10 days or more lose their responsiveness to light and 10 mM KNO_3 to become SD2.
8. DL seeds exposed to light [e.g. daylight fluorescent tubes, 60 μmol/m s (PPFD)] progress to the completion of germination. This can be achieved with a single exposure of the seeds to red light between 20 and 24 h, although shorter exposures and at different times are likely to be just as effective (see Note 22).

3.6. Production of Seeds Undergoing Dormancy Cycling in the Field

3.6.1. Preparing Seeds for Burial

The number of samples to be buried depends on the sampling frequency and duration of the experiment. Seed density, mortality, and substrate are important considerations. Dispersing seeds within a substrate to a known density reduces the seed mortality during prolonged burial as will dressing the seed with fungicide prior to burial (12). Due to the size of *Arabidopsis* seeds, soil is not an appropriate dispersal agent as recovery is difficult. The use of Ballotini balls in the same size range as the soil particulate matter of the field site allows fast and efficient recovery of in excess of 95% of the buried sample. Samples for physiological and molecular determinations can be carried out on the same sample, but separate samples are strongly recommended. The following protocol gives examples for parallel sampling for physiological and molecular determinations of dormancy status in buried seeds.

1. For each bag, attach a tray label to a Weloc clip with wire.
2. Mark each label to denote harvest sequence. If using more than one population/independent biological sample, use different-coloured labels for each.

3. To reduce seed mortality, dress seeds with metalaxyl at 1 g active fungicide/kg seeds prior to dispersal in Ballotini balls (see Note 23).
4. The following quantities should be sufficient to analyze the physiological status of dormancy as described in Subheading 3.4: add 140 mg of seeds to a nylon bag (10×10 cm) containing 85 g of Ballotini balls, seal the bag with a PA110 clip, and mix to disperse the seeds (see Note 24).
5. The following quantities should be sufficient to analyze the molecular status of dormancy: add 50 mg of seeds to bags (7×7 cm) containing 28 g of Ballotini balls and seal each bag with a PA70 clip. For each sample point, bury replicate molecular samples for recovery at the same time. Wire these bags together to aid recovery in the dark (see Notes 25 and 26).

3.6.2. Field Plot Preparation, Maintenance, and Seed Burial

1. Remove weeds from the plot area with a contact herbicide. When weeds have died back, prepare the ground and mark out the plot area. Once the field trial is in place, regularly control invading weeds using a contact herbicide (see Note 26).
2. For each population/independent biological sample, excavate to the desired depth a plot of sufficient area to accommodate the required number of samples. Within this, mark out squares (0.35 m^2), leaving space between each for walkways to aid with exhumation. Mark the centre of each square with a bamboo stake, the top of which is level with the soil surface to indicate the level to backfill with soil. With the dimensions suggested, each quadrant of a square can hold a seed bag for physiological analysis or four smaller seed bags for molecular analysis.
3. Temperature and soil moisture content probes should be located at sowing depth across the burial area to provide data on soil temperature and moisture gradients across the plot area.
4. Use a suitable field plot experimental design to accommodate both physiological and molecular samples and to account for variation across the site (see Note 27).
5. Ensure that the bag contents are mixed well. Lay bags flat on the ground with the contents as a layer of even thickness.
6. Backfill each plot to the level of the bamboo stake; as the soil settles over the next few weeks, these will indicate any additional backfilling.
7. The plot area should be protected from animals by a fence and, if necessary, bag labels can be buried to avoid attracting birds.

3.6.3. Seed Recovery

1. At designated intervals (e.g. monthly), exhume physiological samples for processing (see Note 28).
2. Samples for molecular analysis must be exhumed and processed in the dark (see Note 29).

3. Place the lightproof box over burial site using the depth marker in each square to locate one corner and press down ensuring a lightproof seal (if required, seal gaps externally with soil). The bamboo stake depth marker allows you to locate your position by touch. Using a trowel, carefully uncover the bags. Put the clipped seed bags into the laminated foil bag and seal with a PA150 clip (see Notes 30 and 31).

3.6.4. Separating Seeds in Molecular Samples from Ballotini Balls

The *Arabidopsis* ecotype Cvi has an absolute requirement for light before the germination process can proceed to completion. Light is, therefore, a well-defined signal that removes the final "layer" of dormancy. For this reason, light must be excluded from samples to be analyzed for molecular changes that occur during dormancy cycling in the soil environment.

1. In a darkroom under a green safe light, remove samples from the lightproof bag. Carefully wash each bag in cold water to remove soil. Rinse the Ballotini balls and seeds from each bag into a separate 50-mL centrifuge tube.
2. Let the Ballotini balls and seeds settle and then gently pour off the bulk of the water. Repeat this washing step until water is relatively clear (see Note 32).
3. Gently shake the tube back and forth; seeds migrate over the Ballotini balls to the tube walls.
4. Remove seeds with a wide-mouth pastette and place in labelled Eppendorf tubes (see Note 33).
5. Once all seeds are removed, remove excess water and freeze tubes in liquid nitrogen and store at –80°C.
6. Samples are now ready for molecular analysis.

3.6.5. Separating Seeds in Physiological Samples from Ballotini Balls

1. This step is carried out in normal light. Wash bag in cold water, then open the bag, and wash contents into a glass beaker. Proceed as above, remove seeds with a wide-mouth pastette, and place in a 15-mL centrifuge tube.
2. Proceed to Subheading 3.3 and commence the surface sterilization step before proceeding to Subheading 3.4.

4. Notes

1. These tips fit on to a Gilson P20 pipette and are ideal for plating out small seeded species, such as *Arabidopsis*; the seeds in water can be rapidly and efficiently pipetted onto moist absorbent paper for germination testing.

2. Due to health and safety regulations, pesticides must be handled in accordance with the prevailing regulations and potentially only by a licensed handler.
3. Compost with a nitrogen level of 100 mg N/L is recommended (e.g. Levington F1).
4. Due to health and safety regulations, fungicides must be handled in accordance with the prevailing regulations and potentially only by a licensed handler. As many fungicides have been withdrawn due to concerns over their safety, you may wish to avoid their use. However, severe declines in seed viability may result.
5. Construct nylon mesh bags, 10×10 cm and 7×7 cm. Bags are sewn with polyester thread leaving one side open for filling.
6. In the field, the two most important drivers in dormancy cycling are soil temperature and moisture content, and they must be monitored in the field plot. It is also advisable to measure air temperature.
7. The dormancy characteristics of seed produced under controlled environments are more predictable from generation to generation. Dormancy in seed populations produced under greenhouse conditions is more variable, depending on the time of year regardless of supplementary heating and lighting.
8. A biological seed replicate should have come from a separate mother plant or cohort of mother plants, rather than being a subset of a seed population from a cohort of mother plants. The former gives a greater variation between replicates, but it is a true reflection of the extent of variation. The latter gives greater variation within a replicate seed sample.
9. If unsure as to the presence of residual dormancy, place trays in the dark at 4°C for 2–3 days before transferring to growth conditions.
10. If trying to produce seeds from highly dormant populations or from germination mutants, it may be necessary to break dormancy and germinate seeds on plates first and then transfer seedlings directly to soil.
11. Do not overwater as this encourages Sciarid flies. If they are a problem, water with Intercept systemic insecticide (0.2 g/L) or have it incorporated into your compost.
12. If using other ecotypes, the level of primary dormancy may be lower than that in Cvi; in that case, do not allow plants to completely dry out before harvesting as seeds start to dry afterripen while still on the plant.
13. Surface sterilization dramatically reduces contamination of seeds in germination plates even under non-sterile conditions. It also has the added effect of reducing background germination

probably by removing fungi that release dormancy-breaking compounds, such as permeant weak acids and their derivatives as they attack the seed coat, and enclosing mucilage.

14. Some ecotypes, such as Landsberg *erecta*, germinate rapidly while others, such as Cvi, germinate more slowly.
15. If seeds are limited or if several ecotypes are to be monitored, a minimum of 40 seeds per replicate can be used.
16. It is essential to monitor seed viability at all stages of any field or laboratory study of dormancy in order to rule out declining viability as a cause of reduced germination as dormancy cycles. It also gives an estimate of the longevity of the ecotype, population, or mutant under the prevailing conditions of study. A combination of GA and the ABA biosynthesis inhibitor fluridone provides a robust test of viability. In each test, set up viability plates last and when monitoring germination, score them last to avoid cross-contamination with fluridone.
17. As the depth of dormancy changes in the field, the effective concentration range of GA and the resulting incubation time change in order to elicit a response.
18. The temperature limits for germination are defined as the base (T_b) and ceiling (T_c) temperatures for germination. These are parameters used in the hydrothermal time modelling of germination and dormancy (13, 14). As seeds on the soil surface also experience temperature amplitudes that fluctuate with the season, a range of alternating temperature can also be informative (15).
19. *Arabidopsis* is typically a winter annual exhibiting thermodormancy so that in the absence of dry after-ripening, dormancy is induced at higher temperatures. If a determination of base temperature is required, more frequent temperature intervals will be required. To obtain an estimate of the base temperature, first carry out an initial test on seeds dry after-ripened in the laboratory rather than the ones from the field.
20. In the production of dormant states that require imbibition, imbibe seeds in the germination boxes described, but only use one independent biological replicate per box.
21. In these procedures, all evaluations of germination must be carried out under a green safe light.
22. This is the only step, where seeds are exposed to white light as a treatment.
23. As concerns mount over the safety of some fungicides, with some being withdrawn from use, you may wish to omit this step. However, severe reductions in seed viability may result.

24. A seed density of 40 seeds/g of Ballotini balls is recommended. Check the thousand seed weight before calculating the weight of seeds required.
25. If bags cannot be buried straightaway, they can be stored in the dark at 4°C overnight.
26. Weed growth on the plot may influence the behaviour of buried samples due to the effect of exudates from roots that penetrate the bags.
27. Talk to your local statistician.
28. Remove samples in accordance with the experimental randomized plot design. This requires that an accurate map of the locations of bags, temperature, and moisture probes be maintained. Backfill the resulting hole to prevent the pooling of surface water.
29. *Arabidopsis* seeds are stimulated to germinate by light. In order to obtain a true reflection of molecular changes occurring while seeds are buried, they must be exhumed and processed in darkness on the day of recovery.
30. If the ground is hard or frozen, carefully break up the crust before locating the blackout box. Do not pull on the wires as bags may detach from the clips if the soil is hard or frozen.
31. Two trowels are required at this point for inside and outside the blackout box. To locate the open end of the laminate bag by touch in the blackout box, place the clip inside. Molecular samples should be processed immediately to avoid excessive changes in the transcriptome/proteome.
32. Ballotini balls sediment faster than seeds, resulting in a top layer of seeds. Dead seeds tend to float and are removed in the washing step as are most nematodes that penetrate the bags.
33. At this point, remove any nematodes that have not been removed by the washing step.

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Part III

Hormone Analyses

Chapter 6

Approaches to the Identification of ABAR as an Abscisic Acid Receptor

Xiao-Fang Wang, Qi Xin, Yuan-Yue Shen, and Da-Peng Zhang

Abstract

Abscisic acid (ABA) is a vital phytohormone that regulates seed maturation and germination, seedling growth, and adaptation to environmental stresses. ABA functions through a complex network of signaling pathways, where the cell response is initiated by an ABA receptor which triggers downstream signaling cascades to induce the final physiological effects. Two classes of technologies may be used for the isolation of ABA receptors. One is the genetic screening for ABA receptor mutants, and another is the biochemical isolation of ABA-binding proteins that are putative ABA receptors. We implemented biochemical approaches, namely, the purification of ABA-binding proteins to identify a putative ABA receptor; this protein was further characterized by a combination of biochemical and reverse genetic approaches. The identified ABA receptor, called ABAR, mediates the responses of plants to ABA in seed germination, post-germinative growth, and stomatal movement. This protein is the H subunit (CHLH) of the magnesium protoporphyrin-IX chelatase (Mg-chelatase) that also plays a key role in both chlorophyll biosynthesis and plastid-to-nucleus signaling. Here, we describe the experimental procedures for the purification of ABA-binding proteins and the identification of the ABA-binding protein, ABAR/CHLH, as an ABA receptor.

Key words: Abscisic acid, ABA-binding protein, H subunit of Mg-chelatase, ABA signaling, ABA receptor

1. Introduction

Abscisic acid (ABA) plays a vital role in various aspects of plant growth and development, including seed maturation and germination, seedling growth, and adaptation to environmental stresses (1, 2). ABA functions through a complex network of signaling pathways, where the cell response is initiated by an ABA receptor which triggers downstream signaling cascades to induce the final physiological effects. A receptor for the ABA signal should meet two criteria: (1) an ability to bind specifically to the ABA molecule

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and (2) an ability to mediate ABA-related physiological responses. ABA receptors may be isolated through either genetic or biochemical approaches. Genetic approaches, especially forward genetic screening, have permitted the characterization of numerous downstream components involved in ABA signal transduction, but have failed to identify ABA receptors (2). Biochemical approaches provide another way to isolate ABA receptors by the identification of ABA-binding proteins that are putative ABA receptors. We undertook a biochemical approach to isolate ABA-binding proteins and purified an ABA-binding protein (ABAR, for putative ABA receptor) via affinity chromatography from broad bean (*Vicia faba*). We showed that the broad bean ABAR was potentially involved in ABA-mediated guard cell regulation (3). The ABAR protein was identified by sequencing as the H subunit (CHLH) of the magnesium protoporphyrin-IX chelatase (Mg-chelatase) (4), which is a key component in both chlorophyll biosynthesis and plastid-to-nucleus signaling (5). Further characterization of the ABAR/CHLH ortholog of *Arabidopsis* demonstrated that it is a bona fide ABA receptor; it specifically binds ABA and mediates ABA signaling as a positive regulator in seed germination, postgerminative growth, and stomatal movement (4, 6). This is a good example to show that the isolation of ABA-binding proteins by use of carefully designed affinity probes is efficient in the identification of putative ABA receptors, and further shows the power of subsequently combining biochemical and genetic approaches to unequivocally prove that a protein is a receptor. Multiple receptors may exist for ABA signal perception (7). Thus, the identification of novel ABA receptors continues to be important for uncovering the complex and diverse ABA-signaling mechanisms underlying physiological processes. Recent studies identified a plasma membrane and an intracellular receptor for ABA by using reverse genetic approaches (8–10). Forward genetic screens with improved screening strategies may be auspicious for the isolation of new ABA receptor mutants. However, efforts involving biochemical approaches are still useful in screens for distinct ABA receptor candidates. In this section, we describe the experimental procedures for the purification of ABA-binding proteins and the identification of ABAR/CHLH as an ABA receptor.

2. Materials

2.1. Plant Materials

1. Broad bean (*V. faba*) plants: These are grown in pots filled with soil composed of loam, peat, and coarse sand in a 7:3:2 volume ratio, supplemented with nitrogenphospho-potash complete chemical fertilizer and maintained in a greenhouse under a 14-h photoperiod with day/night temperatures of

25/18°C (light intensity approximately 300 μmol/m²/s). The plants are watered once a day and used for experiments when they are 4 weeks old.

2. *Arabidopsis thaliana* ecotypes gl1 and Columbia: gl1 is *A. thaliana* ecotype Columbia carrying the homozygous recessive *glabrous* mutation. The two ecotypes are used for the generation of transgenic plants (4, 6). The plants are grown in a greenhouse under a 16-h photoperiod with day/night temperatures of 20/18°C (light intensity approximately 120–150 μmol/m²/s).

2.2. Extraction and Purification of ABA-Binding Proteins

1. Immersion buffer for leaves: 10 mM MES (Amresco), pH 6.0 (adjusted with NaOH), 0.02% (w/v) bovine serum albumin (BSA), 0.25 M mannitol, 0.1 mM $CaCl_2$, 1 mM ethylene glycol-bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA, Amresco).
2. Grinding buffer: 10 mM MES pH 6.5 (adjusted with NaOH), 100 mM NaCl, 1 mM $MgCl_2$, 2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride (PMSF, Amresco), 2 mM dithiothreitol (DTT), 0.5% (v/v) Triton X-100 (Amresco).
3. Washing buffer I: 0.5 M NaCl, 0.1 M Tris (Amresco), pH 8.3 (adjusted with HCl).
4. Washing buffer II: 0.5 M NaCl, 0.1 M sodium acetate-acetic acid, pH 4.0.
5. Buffer A: 10 mM MES pH 6.5 (adjusted with NaOH), 150 mM NaCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM KCl.

2.3. SDS-Polyacrylamide Gel Electrophoresis

1. Separating buffer: 1.5 M Tris, pH 8.8 (adjusted with HCl). Store at room temperature.
2. Stacking buffer: 0.5 M Tris, pH 6.8 (adjusted with HCl). Store at room temperature.
3. Acrylamide/bis solution: 29 g acrylamide (Fluka), 1 g bis-acrylamide (Fluka), 100 mL distilled water, 0.22 μm filtered.
4. Running buffer (5× stock): 125 mM Tris, 960 mM glycine, 0.5% (w/v) sodium dodecyl sulfate (SDS, Amresco). Store at room temperature.
5. Staining solution: 0.5 g Coomassie Brilliant Blue R-250 (Amresco), 50 mL acetum, 200 mL methanol, 250 mL distilled water.
6. Destaining solution: 50 mL acetum, 200 mL methanol, 250 mL distilled water.

2.4. IEF/SDS-PAGE Two-Dimensional Electrophoresis

1. IEF buffer: 9.5 M urea (Amresco), 2% (v/v) Triton X-100, 5% (v/v) β-mercaptoethanol (Amresco), 1.6% (v/v) ampholyte, pH 5.0–7.0, 0.4% (v/v) ampholyte, pH 3.0–10.0.

2. IEF tube gel: 9.2 M urea, 4% (w/v) acrylamide, 2% (v/v) Triton X-100, 1.6% (v/v) ampholyte, pH 5.0–7.0, 0.4% (v/v) ampholyte, pH 3.0–10.0.
3. SDS sample buffer: 62.5 mM Tris, pH 6.8 (adjusted with HCl), 2.3% (w/v) SDS, 5.0% (v/v) β-mercaptoethanol, 10% (v/v) glycerol.

2.5. Extraction of Total Protein of Arabidopsis

1. Extraction buffer I for immunoblotting: 100 mM Hepes (Amresco), pH 8.0, 5 mM EGTA, 5 mM ethylenediaminetetraacetic acid disodium salt(EDTA Na_2, Amresco), 10 mM DTT, 10 μg/mL leupeptin (Amresco), 10 μg/mL aprotinin (Amresco), 10 μg/mL antipain (Amresco), 1 mM PMSF, 5% (v/v) glycerol.
2. Extraction buffer II for ABA-binding assays: 50 mM Tris, pH 7.0 (adjusted with HCl), 2 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM DTT, 1 mM PMSF, 50 μg/mL leupeptin, 50 μg/mL pepstatin A (Amresco).
3. Extraction buffer III for pull-down assays: 50 mM Tris, pH 7.0 (adjusted with HCl), 250 mM mannitol, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM DTT, 1 mM PMSF, 50 μg/mL leupeptin, 50 μg/mL pepstatin A.

2.6. Immunoblotting

1. Transferring buffer: 25 mM Tris, 192 mM glycine (Amresco), 20% (v/v) methanol. The pH of this mixture is at 8.3. This is prepared fresh and stored at 4°C just before use.
2. Blocking buffer and primary antibody dilution buffer: 10 mM Tris, pH 7.5 (adjusted with HCl), 150 mM NaCl, 3% (w/v) BSA, 0.05% (v/v) Tween-20 (Amresco).
3. Tris-buffered saline 1 with Tween-20 (TBST1): 10 mM Tris, pH 7.5 (adjusted with HCl), 150 mM NaCl, 0.05% (v/v) Tween-20.
4. Tris-buffered saline 2 with Tween-20 (TBST2): 50 mM Tris, pH 7.5 (adjusted with HCl), 150 mM NaCl, 0.1% (v/v) Tween-20.

2.7. In-Buffer ABA-Binding Assay

1. The binding medium: 50 mM Tris, pH 7.0 (adjusted with HCl), 2 mM $MgCl_2$, 1 mM $CaCl_2$, 250 mM mannitol.

2.8. Physiological Experiments

1. Epidermal incubation buffer for stomatal observation: 10 mM MES, pH 6.15 (adjust with KOH), 50 mM KCl.

3. Methods

3.1. General Consideration

As mentioned in the Introduction, the procedures for the isolation of an ABA receptor (such as ABAR/CHLH) can be outlined under

two major steps. The first step is the isolation of the ABA-binding protein; and in our discussion, we use the example of the broad bean ABAR. The second step is the demonstration of an ABA-binding protein as an ABA receptor. For this step, we describe the identification of the *Arabidopsis* ABAR (AtABAR) as an ABA receptor. The successful isolation of ABA-binding proteins is a key step for the biochemical approaches to identify ABA receptors. The complete procedures of the isolation of the ABA-binding protein, ABAR, from broad bean can be summarized as follows: (1) extraction of proteins from leaves with analysis of ABA-binding; (2) protein purification using an affinity chromatography column with analysis of ABA binding; (3) analysis of the purified protein with SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and IEF/SDS-PAGE two-dimensional electrophoresis; (4) sequencing of the ABA-binding protein, ABAR, after purity has been demonstrated by IEF/SDS-PAGE two-dimensional electrophoresis; (5) isolation of the gene coding for the ABAR protein based on the ABAR sequence information; (6) expression of the *ABAR* gene in a yeast or *E. coli* system; and (7) confirmation of the ABA-binding activity of the expressed ABAR protein.

For the isolation of ABA-binding proteins, use of the appropriate plant tissues with concentrated ABA-binding proteins and a suitable affinity chromatography medium is of primary importance for an efficient purification. The ABA-linked EAH-Sepharose 4B was used as the affinity medium to purify the broad bean ABAR (3). EAH-Sepharose 4B possesses a 10-atom spacer arm that may be able to provide a better spacing effect for the efficient reaction of ABA-binding proteins with the coupled ABA to this arm. Such an ABA-linked EAH-Sepharose 4B and a high coupling efficiency of ABA to this gel (about 80%) may greatly improve the purification efficiency (3). Also, an efficient extraction, an optimum pH during extraction and purification, and an appropriate concentration of NaCl used for elution are all important for the purification, which should ensure that the ABA-binding proteins are as highly enriched as possible in the isolation buffers on the one hand; and on the other hand, the isolated ABA-binding proteins retain their maximum binding activity to ABA.

In order to successfully isolate proteins with bona fide ABA-binding abilities, it is crucial to implement an efficient technique for detecting ABA-binding activity. The different ABA-binding proteins may respond to environmental or developmental cues by binding ABA under different conditions, so the ABA-binding detection systems may be different for distinct ABA-binding proteins: a system adapted well to one ABA-binding protein may not be necessarily suited to other ABA-binding proteins.

In regard to this, we have noted that the paper reporting FCA as an ABA receptor (11) has recently been retracted (12) mainly because FCA does not bind ABA by using an in-buffer binding system (12, 13). However, the authors initially used this in-buffer

system to detect the ABA binding to FCA (11). This indicates that the in-buffer binding system is difficult to pursue. To avoid errors associated with assessing the ABA-binding capabilities of a known protein, we recommend that, in addition to being used in the purification of unknown ABA-binding proteins as described below (see Subheadings 3.3 and 3.4), the ABA-linked Sepharose 4B should also be used to assess qualitatively whether a given protein binds ABA. The purified yeast- or *E. coli*-expressed protein is loaded onto the affinity column that is first eluted with suitable concentrations of NaCl to remove the nonspecifically bound proteins and is then eluted with the buffer containing ABA. The proteins in the different eluting fractions are determined by immunoblotting. The nature of the ABA binding can be identified for this protein if the protein can be eluted by an appropriate concentration of ABA after the nonspecific binding portion is completely eliminated through the intensive washing with NaCl. The ability of the protein to bind ABA can be further confirmed qualitatively by loading the crude extracts prepared from plant tissues onto the affinity column and assaying the ABA-binding ability with the same procedures of elution and immunoblotting as mentioned above. This method is simple and reliable and is easily repeated. We have obtained satisfactory results with this method to confirm the ABA-binding nature of the ABAR/CHLH protein (6). The pull-down assay described below may be a supplementary assay to confirm the ABA-binding ability of the protein.

In addition to the qualitative assays of ABA binding, the kinetics of the ABA binding must be subsequently determined. The in-buffer binding allows a characterization of the ABA-binding kinetics, including mainly the binding affinity and density (maximum binding) (see Subheading 3.9). A set of suitable procedures for the in-buffer binding assay must be carefully established for a given protein which may be more or less different for distinct proteins as mentioned above. The central principle is to reduce as much as possible the unspecific binding while enhancing the total binding. The unspecific binding must be less than or around 10% of the total binding for an efficient binding-detection in-buffer system. To meet this, the in-buffer conditions must be assayed extensively so that the best conditions can be determined (Subheading 3.9, also see Notes 4–8).

The procedures for the identification of the AtABAR as an ABA receptor can be summarized as two major steps: the first involves an assessment of the ABA-binding abilities of AtABAR using both the in-buffer binding system and pull-down assays; the second involves a functional analysis of AtABAR in mediating ABA signaling during physiological processes, such as seed germination, seedling growth, and stomatal movement. This latter step is achieved through the transgenic/genetic manipulation of the down- or up-expression of the *ABAR* gene in *Arabidopsis*.

These procedures involve both the biochemical and genetic approaches. We present below some biochemical and physiological methods that we consider important for this research, but do not include standard methods used to transform plants since these are well-described in plant biology literature. We recommend the review of (3) and (4) for further information.

3.2. Extraction of ABA-Binding Proteins

1. The leaves of broad bean are harvested from 4-week-old plants. The lower epidermis is peeled off from the leaves and immersed for 3–4 h in the cold immersion buffer at 4°C. The epidermis samples are either kept at 0°C for immediate use or frozen in liquid nitrogen and stored at −80°C until use.
2. The epidermis sample is ground with a pestle and mortar in the grinding buffer with a sample weight: grinding buffer volume ratio of 1:3.
3. Centrifuge for 15 min at 15,000 × *g*.
4. The supernatant is centrifuged again at 100,000 × *g* for 30 min.
5. The supernatant is concentrated to 3–4 mg protein/mL by ultrafiltration.

3.3. Preparation of ABA-Linked EAH-Sepharose 4B

ABA is linked to EAH-Sepharose 4B via C1 (−COOH) of the ABA molecule (3). This ABA-linked Sepharose may be used not only for the purification of ABA-binding proteins, but also for the analysis of the ABA-binding ability of proteins (as described above in Subheading 3.1).

1. (±)ABA (1 g, Sigma) dissolved in 60 mL of 50% (w/v) dimethylformamide (DMF) solution is mixed with 50 mL of drained EAH-Sepharose 4B (GE healthcare).
2. 1-ethyle-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (4 g, EDC, Sigma) is added to the ABA–EAH-Sepharose 4B solution, of which the pH is adjusted to 8.0 with 1 N NaOH.
3. The ABA–EAH-Sepharose 4B solution is shaken for 20 h at 4°C in the dark.
4. The ABA–EAH-Sepharose 4B gel is washed with 50% (w/v) DMF and then is washed sequentially with washing buffer I and washing buffer II.
5. The gel is washed with double-distilled water.
6. The coupling amount of ABA to EAH-Sepharose 4B is determined essentially according to Nilsson and Mosbach (14). ABA–EAH-Sepharose 4B (40 mg) is dissolved in 80% (v/v) glycerol, and then the UV A252 of the solution is measured with an UV-Photometer (UV-240, Shimandz, Tokyo) using 80% (v/v) glycerol as the control. The amount of the coupled ABA is calculated according to the standard UV absorbance

per millimolar ABA at 252 nm. The tested coupling efficiency, which is referred to as the ratio of the coupling amount of ABA to the total amount of the amino groups conjugated to the gel, is approximately 80%.

3.4. Purification of ABA-Binding Proteins

1. The ABA–EAH-Sepharose 4B gel is packed into a column of 1.6 × 50 cm for affinity chromatography. This affinity column is equilibrated with the buffer A solution (see Note 1).
2. The crude extract obtained with Triton X-100 as the extraction medium is loaded onto the affinity column.
3. The column is first eluted with a step gradient of NaCl in buffer A (1,000 mL), from 100 to 300 mM, to remove any proteins that are nonspecifically bound to the column.
4. ABA-binding proteins are then eluted with the same buffer A containing 1 mM (±) ABA (300 mL). The eluting solutions are assayed for ABA-binding activity, and all the eluting fractions containing ABA-binding activity are pooled.
5. The free ABA in the eluting solution is removed by passing the solution through a Sephadex G-25 column.
6. The eluting solution is concentrated by ultrafiltration.

3.5. SDS-PAGE for Analysis of ABA-Binding Proteins

1. The SDS-PAGE of the purified ABA-binding proteins is carried out essentially according to Laemmli (15) with the Mini-PROTEAN II System (Bio-Rad). For a 12% gel, mix 2.5 mL separating buffer with 4 mL acrylamide/bis solution, 3.35 mL water, 100 µL 10% (w/v) SDS, 50 µL of 10% (w/v) ammonium persulfate solution, and 5 µL *N*,*N*,*N*′,*N*′-Tetramethylethylenediamine (TEMED, Ameresco). In contrast to the 12% gel, the 7.5% gel should contain 2.5 mL acrylamide/bis solution and 4.85 mL water (see Note 2). Pour the gel, leave space for a stacking gel, and overlay with distilled water. The gel should polymerize in about 60 min.
2. Blot up the water between the two layers of glass plates with filter paper.
3. Prepare the stacking gel by mixing 1.25 mL of stacking buffer with 0.67 mL acrylamide/bis solution, 3.0 mL water, 50 µL 10% (w/v) SDS, 25 µL 10% (w/v) ammonium persulfate solution, and 5 µL TEMED. Pour the stacking gel and insert the comb. The stacking gel should polymerize within 30 min.
4. Prepare the running buffer by diluting 80 mL of the 5× running buffer with 320 mL of water in a measuring cylinder.
5. Remove the comb when the stacking gel has polymerized.
6. Add the running buffer to the gel unit and load each sample in a well.

7. Complete the assembly of the gel unit and connect to a power supply. Run the gel for 30 min at 80 V and then for about 1 h at 150 V.
8. When the dye front runs off the gel, turn off the power supply and detach the glass plates.
9. Stain the gel with staining solution for 15 min and then destain with destaining solution.

3.6. IEF/SDS-PAGE Two-Dimensional Electrophoresis for Analysis of ABA-Binding Proteins

The pI of the purified ABA-binding protein can be determined with IEF using the Model III Mini-IEF System (Bio-Rad) on polyacrylamide gels containing ampholytes (pH 3.0–10.0). Two-dimensional electrophoresis IEF/SDS-PAGE is performed essentially according to O'Farrell (16) with the Mini-PROTEAN II 2-D System (Bio-Rad). Samples are run from the cathodic reservoir (100 mM NaOH) to the anodic reservoir (10 mM H_3PO_4).

1. A pre-electrophoresis step is conducted at 200 V for 10 min, 300 V for 15 min, and 400 V for 15 min, and the ABA-binding protein is dissolved in the IEF buffer.
2. Load the dissolved sample into the IEF tube gel.
3. The IEF is conducted at 500 V for 10 min and at 750 V for 3.5 h.
4. After the electrophoresis, the tube gel is equilibrated with SDS sample buffer and then applied to a 12% (v/v) SDS-PAGE gel for electrophoresis in the second dimension.
5. Proteins in the gel are detected by silver staining using the Silver-Staining Kit of Protein (Amersham Pharmacia).

3.7. Extraction of the ABAR/CHLH Protein

1. The leaves of *Arabidopsis* are harvested from 3-week-old plants and ground in liquid nitrogen.
2. When transferring the ground materials into an Eppendorf tube, cold extraction buffer I (1 mL/g sample) is added and proteins are extracted for 2–3 h on ice.
3. The extract is centrifuged for 20 min at 16,000 × *g* and the supernatant is collected.
4. The supernatant is centrifuged again at 16,000 × *g* for 20 min, and then the protein concentration of the supernatant is determined by Coomassie Brilliant Blue G-250 (Amresco).
5. The samples are either kept at 0°C for immediate use or frozen and stored at –80°C until use.

3.8. Immunoblotting of the ABAR/CHLH Protein

The immunoblotting of the purified ABA-binding proteins or ABAR/CHLH is carried out according to the methods used in our laboratory. Electrophoretic transfer of polypeptides from SDS-polyacrylamide gels to nitrocellulose membranes (0.45 μm,

Amersham Life Science) is conducted with a Mini Trans-Blot cell (Bio-Rad).

1. When SDS-PAGE is over, the proteins on the mini gel are transferred to a membrane in transferring buffer for 10 h (100 mA) at 4°C.
2. The membrane is blocked for 2 h at room temperature with blocking buffer/primary antibody dilution buffer.
3. The membrane is incubated with gentle shaking for 2 h at room temperature in the anti-ABAR N-terminus ($ABAR^N$) serum (see Note 3).
4. The membrane is washed three times for 10 min each in the TBST1 buffer.
5. The membrane is then incubated with the alkaline phosphatase-conjugated secondary antibody raised in goat against mouse IgG (diluted 1:1,000 in the blocking buffer) at room temperature for 1 h.
6. The membrane is washed three times for 10 min each with the TBST2 buffer.
7. Protein bands are visualized by incubation in the 5-bromo-4-chloro-3-indolyl-P/nitroblue tetrazolium substrate system.

3.9. ABA-Binding System: In-Buffer Assay

The in-buffer ABA-binding assay is conducted according to Zhang et al. (3). The concentration of [^{3}H]-(+)ABA (American Radiolabeled Chemicals, 2.37×10^{12} Bq/mmol, purity 98.4%) is 30 nM for 2 μg of purified proteins (e.g., purified yeast-expressed AtABAR/CHLH protein or purified broad bean ABAR) or 50 μg of crude extract proteins (e.g., derived from 2-week-old *Arabidopsis* seedlings or broad bean leaves) (see Note 4). A step gradient of concentrations of [^{3}H]-ABA from 0 to 70 nM is used when analyzing ABA-binding kinetics. Total proteins are extracted from 2-week-old *Arabidopsis* seedlings with extraction buffer II.

1. Incubate the mixtures of proteins and [^{3}H]-ABA in the binding medium (see Note 5) at 25°C for 30 min. The binding may be conducted at 4°C for a duration of 60 min, which may be prolonged for up to 6 h or even to 8 h according to the time course of the protein activity to bind ABA (3) (also see Note 6). The total volume for the binding assays is adjusted to 200 μL.
2. The tubes containing the reaction mixture are placed on ice quickly.
3. Following the addition of 50 μL 0.5% (w/v) Dextran T70-coated charcoal (DCC) for crude ABA-binding proteins or 100 μL for the purified ABAR to remove the free [^{3}H]-ABA by adsorption (see Note 7), the mixtures are maintained on ice for 10 min.

4. The samples are centrifuged at approximately 150 × *g* for 1 min to remove the DCC before the radioactivity in the supernatant is counted.
5. The radioactivity of the sample that includes 5 μL of supernatant and 5 mL of scintillation fluid is counted using a liquid scintillometer (LS-5801, Beckman Instruments, Fullerton, CA). The specific binding is determined by the difference between the radioactivity bound to the purified ABAR protein or crude extracts incubated only with [^{3}H]-ABA (total binding) and the radioactivity bound in the presence of 1,000-fold molar excess of unlabeled (±)-ABA (unspecific binding) (see Note 8). The unlabeled ABA is added into the incubation medium at the same time with [^{3}H]-ABA. The ABA-binding activity of the ABA-binding protein of the crude extracts is expressed as the number of nanomoles of [^{3}H]-ABA specifically bound per gram of protein, and that of the purified ABAR protein as the number of moles of [^{3}H]-ABA per mole of protein.

3.10. ABA-Binding System: Pull-Down Assay

The pull-down assay can be used to detect ABA binding to the natural ABAR protein extracted from plants. Total proteins are extracted from 2-week-old *Arabidopsis* seedlings with extraction buffer III.

1. The crude extract (3 mg of total proteins) is incubated by gently shaking in 400 μL of extraction buffer III containing 30 nM [^{3}H](+)ABA at 4°C for 2 h.
2. 10 μg of anti-ABAR^N serum is added for a further incubation at 4°C for 2 h.
3. 100 μL of 25% (v/v) protein G-agarose (Santa Cruz) is added for a final incubation at 4°C for 2 h.
4. Following the incubation, protein G-agarose is recovered by a brief centrifugation (at 1,000 × *g* for about 15 s) and washed three times with 1 mL of extraction buffer III.
5. The pellet is resuspended in 100 μL of water and mixed with 5 mL of scintillation fluid before the radioactivity (disintegrations per minute, d.p.m.) of the bound [^{3}H]-ABA is measured (see Note 9).

3.11. Physiological Experiments

The physiological assays are conducted for the identification of ABAR as an ABA-signaling regulator in part by observing the phenotypes of mutants in which the *ABAR* gene (or another related gene) is overexpressed or down-regulated.

1. For the germination assay, approximately 100 seeds each from wild types and mutants or transgenic mutants are surface-sterilized and planted in triplicate on Murashige and Skoog (MS) media (Sigma) containing different concentrations of (±)-ABA

(0, 0.1, 0.5, 1, 3 μM) and incubated at 4°C for 3 days before being placed at 20°C under light conditions. Germination (emergence of the radicle) is scored after 24 h.

2. For the seedling growth experiments, seeds are germinated after moist chilling ("stratification") on common MS medium. Forty eight hour later, seeds are transferred to MS media supplemented with different concentrations of ABA (0, 0.1, 0.5, 1, 5, 10, 20, 40, 80, 100 μM) in the vertical position. Seedling growth is observed 10 days after the transfer (see Note 10).
3. For the stomatal aperture assays, 3-week-old leaves are used. To observe ABA-induced closure, leaves are floated in the epidermal incubation buffer under a halogen cold-light source (Colo-Parmer) at 200 μmol/m^2/s for 2 h followed by addition of different concentrations of (±)-ABA. Apertures are recorded on epidermal strips after 2 h of further incubation to estimate effects on stomatal opening. To observe inhibition of stomatal opening, leaves are floated on epidermal incubation buffer in the dark for 2 h before they are transferred to the cold light (200 μmol/m^2/s) for 2 h in the presence of ABA, and then apertures are recorded.
4. For the drought tolerance experiment, plants are grown aseptically in Petri dishes containing selective agar germination medium for 2 weeks and then transferred to 8-cm compost-soil-filled pots. Fifteen days later, when plantlets have reached the stage of five to six fully expanded leaves, drought is imposed by withdrawing irrigation for one-half of the plants until lethal effects are observed for most of the stressed plants. The remaining plants are grown under a standard irrigation regime and serve as controls.
5. For the assay of water loss from leaves, 3-week-old rosette leaves are detached from their roots, placed on filter paper, and left on the lab bench. The loss in fresh weight is monitored once with an interval of 30 min.

4. Notes

1. Unless otherwise indicated, all of the procedures described for the purification of ABA-binding proteins are conducted at 4°C.
2. The concentration of the running slab gel was 12% for the broad bean ABA-binding protein and 7.5% for the AtABAR/CHLH.
3. A fragment of ABAR/CHLH cDNA corresponding to the N-terminal 258 amino acids (from amino acid 52 to 310) was

expressed in *E. coli* as a glutathione S-transferase-ABAR^{N} fusion protein. The antiserum diluted with the blocking buffer may be stored at 4°C for 2–3 weeks. The affinity-purified fusion protein was used for standard immunization protocols in mice (4).

4. Ensure that the protein sample has its maximum ABA-binding activity, which may be affected by various conditions. A fresh sample of the proteins is essential to preserve the optimum binding activity while freeze thawing has an adverse effect on the binding activity.
5. Determine an appropriate binding buffer with the optimum pH. This generally varies with proteins from about pH 6.5–7.0.
6. The time course and temperature dependence of ABA-binding activity must be determined for a given ABA-binding protein which may change according to different proteins and various conditions in the binding detection system. The principle to determine the best binding duration and optimum medium temperature is to try to maintain the maximum (or a high level) of the specific binding in the specific binding-detecting system implemented for a given protein (3).
7. The quality of the DCC should be tested before use in binding assays. The DCC should not pull the proteins (or protein-[^{3}H]-ABA complexes) down when removing free [^{3}H]-ABA. The concentrations of the DCC used may be varied from 0.5 to 5% according to the binding conditions of the proteins to be assayed. The total amount of DCC used for removing free [^{3}H]-ABA should be carefully determined so that the total binding attained is at the maximum while the unspecific binding is reduced to a minimum (i.e., less than 10% of the total binding). Alternately, a filter can be used in the ABA-binding assay to remove free [^{3}H]-ABA from the binding medium instead of using DCC (please see ref. 6 for the detail).
8. The amounts of unlabeled ABA used for removing the specific ABA binding to the proteins may be varied from 100-fold to 1,000-fold [^{3}H]-ABA according to the binding conditions of the proteins to be assayed. The same principle as described above for determining the DCC amounts is also applied to determine the amounts of unlabeled ABA to be used in the binding assay, which must ensure that the total binding attained is at the maximum while the unspecific binding is reduced to a minimum. The suitable combination of the unlabeled ABA and DCC should be selected for an efficient in-buffer binding system.
9. Three independent control experiments were performed for the pull-down assays. A control was conduced by addition of the same amounts of the mouse preimmune serum in place of the antiserum to the medium of the pull-down assay in the

extracts from the leaves of wild-type plants. In the second control experiment, [^{3}H]-ABA binding was assayed in the binding medium as described in Subheading 3.10 with the supernatants obtained after the precipitation of the wild-type plant extracts with the preimmune serum at 4°C for 2 h. Depleting ABAR protein with the anti-ABAR serum from the wild-type plant extracts was taken as the third control. The anti-ABAR serum (20 μg) was added to the extracts for incubation at 4°C for 2 h to deplete ABAR protein from the total proteins, and the supernatants deprived of ABAR protein were obtained after removing protein-G-agarose-antiserum/ABAR protein complexes by centrifugation. These supernatants were used to assay either [^{3}H]-ABA binding or residual ABAR protein by immunoblotting with anti-ABAR^N serum.

10. The seedlings should be transferred to the ABA-containing medium less than 48 h after moist chilling (stratification). The status of the seedlings is characterized by the nongreen cotyledons when transferred to the ABA-containing medium.

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Chapter 7

Profiling of Hormones and Related Metabolites in Seed Dormancy and Germination Studies

Mitsunori Seo, Yusuke Jikumaru, and Yuji Kamiya

Abstract

Seed dormancy and germination are regulated by several plant hormones, such as abscisic acid, gibberellin, auxin (indole-3-acetic acid), ethylene, and brassinosteroid. Endogenous concentrations of a hormone are determined by the balance between biosynthesis and deactivation, and contribute to the regulation of physiological responses. Therefore, profiling of all hormones and their metabolites (hormonome) is a powerful approach to elucidate the regulatory networks of hormone metabolism. The methods involved in the use of liquid chromatography–electrospray ionization–tandem mass spectrometry to develop a high-sensitive and high-throughput hormonome platform are described in this chapter.

Key words: Dormancy, Germination, Hormone interaction, Hormone profiling, Liquid chromatography–electrospray ionization–tandem mass spectrometry, Plant hormones

1. Introduction

It is well-known that the plant hormone abscisic acid (ABA) is involved in the induction and maintenance of seed dormancy (reviewed in ref. 1–3). However, dormancy is not merely regulated by ABA alone, but rather by the combination or interaction with other hormones. Gibberellin (GA) acts on seed dormancy antagonistically to ABA, i.e., GA promotes seed germination (1–3). Recent studies clearly demonstrate that metabolism of ABA and GA is reciprocally regulated by each other (4). Here, we refer to biosynthesis as the production of bioactive forms of a hormone while deactivation refers to the conversion of bioactive forms (or their precursors) to the inactive or less active forms. Thus, metabolism refers to both biosynthesis and deactivation. Other hormones, such as auxin (indole-3-acetic acid; IAA), ethylene, and brassinosteroid (BR), have also been implicated in the regulation of dormancy and/or germination (5–9). Since the physiological

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actions of a hormone depend on its endogenous concentrations and the ability of the cell to respond to the hormone, it is important to determine endogenous hormone levels precisely. The balance between biosynthesis and deactivation determines endogenous concentrations of a hormone. Thus, to understand detailed regulatory mechanisms that control hormone levels in plants, it is important to analyze the levels of hormone metabolites as well–both hormone precursors and deactivated forms of a hormone.

Plant hormones exist in plant tissues at much lower concentrations than those of primary and secondary metabolites; generally, hormones are present in pico-nano gram orders per gram dry weight as compared to micro-milli gram orders per gram dry weight for primary/secondary metabolites. Gas chromatography–mass spectrometry (GC–MS) has been commonly used for the quantification of plant hormones. Analysis with GC–MS requires complicated purification steps, including stepwise solvent partitioning and high-performance liquid chromatography (HPLC). Derivatization of the target compounds is also required if they are not volatile. These procedures differ depending on targets to be analyzed and are optimized for each hormone (10–16).

Liquid chromatography–electrospray ionization–tandem mass spectrometry (LC–ESI–MS/MS) is a powerful tool that has allowed for the development of a highly sensitive and high-throughput hormone analysis platform (8, 17–20). This method is suitable for analyzing several (or potentially all) hormones and their metabolites simultaneously. LC–ESI–MS/MS consists of three components: LC, ESI system, and MS/MS (Fig. 1a). Partially purified samples are first separated on LC in combination with various columns (see Note 1) and gradients of mobile phase composition. Target molecules separated on LC have to be ionized by the second component ESI to be introduced into the last component MS/MS (Fig. 1b). Liquid eluted from LC is charged by passing it through a charged capillary (21). If the capillary is charged negatively, for example, liquid containing water (H_2O) is charged negatively as a consequence of hydroxide (OH^-) generation. Droplets of charged liquid sprayed from the capillary are concentrated at a high temperature, resulting in the breakup of droplets by the repulsion among negative ions. The target molecules (in this case, acidic compounds such as carboxylic acids) can be charged negatively by the removal of a proton from a functional group due to the reaction with OH^-. The ionized target molecules are introduced into the MS/MS detection unit according to electric charge (Fig. 1). We have employed two types of MS/MS, i.e., Q (quadrupole)-Q type (Agilent 6410, Agilent) and quadrupole time-of-flight (Q-Tof) type (Q-Tof premier, Waters). In each system, molecular ions produced by ESI are selected by passing through the first MS quadrupole based on desired m/z (e.g., 263 for ABA; Fig. 1a) (22). Selected protonated/deprotonated molecules are then fragmented into specific patterns by the collision-induced dissociation (CID) system.

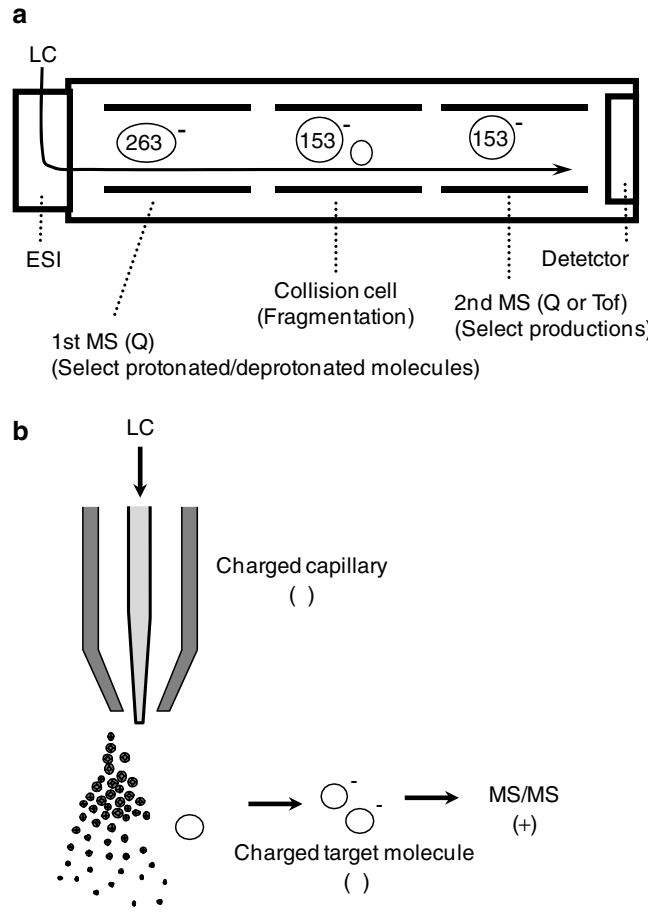

Fig. 1. (**a**) Composition of LC–ESI–MS/MS. Detection of ABA (deprotonated molecules *m/z* 263 and product ion *m/z* 153) is illustrated as an example. (**b**) Ionization of the target molecule (in this case, an acidic compound) by ESI and introduction into MS/MS are illustrated.

The product ions are selected based on *m/z* (e.g., 153 for ABA; Fig. 1a) (22) by the second MS (Q or Tof) and finally detected by the detector. Using an LC–ESI–MS/MS system, several hormones and hormone metabolites can be separated directly on LC and analyzed by MS/MS by single injection. This is a key advantage of this system, and is conducive to highly sensitive and high-throughput hormonome analysis.

2. Materials

2.1. Sample Extraction and Purification

1. Methanol (MeOH).
2. Chloroform.
3. Acetonitril (MeCN).

4. 0.1 M HCl.
5. 0.1 M NaOH.
6. Eighty percent MeOH containing 1% acetic acid.
7. Eighty percent MeOH containing 1% formic acid.
8. Sixty percent MeOH containing 5% aqueous ammonia.
9. Water containing 1% acetic acid.
10. Water containing 5% aqueous ammonia.
11. Chloroform:MeOH, 9:1 (v/v).
12. Oasis HLB (Waters, Milford, MA, USA).
13. Oasis MCX (Waters).
14. Oasis WAX (Waters).
15. SepPak silica (Waters).
16. Internal standards: D_6-ABA (ICON ISOTOPES, Summit, NJ, USA); D_2-GA_1, D_2-GA_4, D_5-tZ, D_3-DHZ, D_6-iP (Olchemim Ltd, Olomouc, Czech); D_2-IAA, D_6-SA (SIGMA-ALDRICH, Oakville, ON, Canada); D_2-JA (Tokyo Kasei, Tokyo, Japan).
17. Polyvinylpyroridone (PVP) (if required).
18. *n*-Hexane (if required).
19. Ethyl acetate (if required).

2.2. LC–ESI–MS/MS Analysis

1. ACQUITY UPLC system (Waters).
2. Q-Tof premier (Waters).
3. ACQUITY UPLC BEH C18, 2.1 × 50 × 1.7 mm (Waters).
4. Agilent 1200 (Agilent, Santa Clara, CA, USA).
5. Agilent 6410 (Agilent).
6. ZORBAX Eclipse XDB-C18, 2.1 × 50 × 1.8 mm (Agilent).
7. Spectrometer software (MassLynx™ v. 4.1, waters or MassHunter™ v. B. 01. 02, Agilent).
8. Water containing 0.01% acetic acid.
9. MeCN containing 0.05% acetic acid.
10. MeCN containing 0.1% formic acid.
11. Formic acid, 0.1%.

3. Methods

Here, we describe a fundamental procedure to quantify hormones for which stable isotope-labeled standards are commercially available: ABA, GA_1, GA_4, IAA, *trans*-zeatin (tZ), dihydrozeatin (DHZ), isopentenyl adenine (IP), salicylic acid (SA), and jasmonic acid (JA).

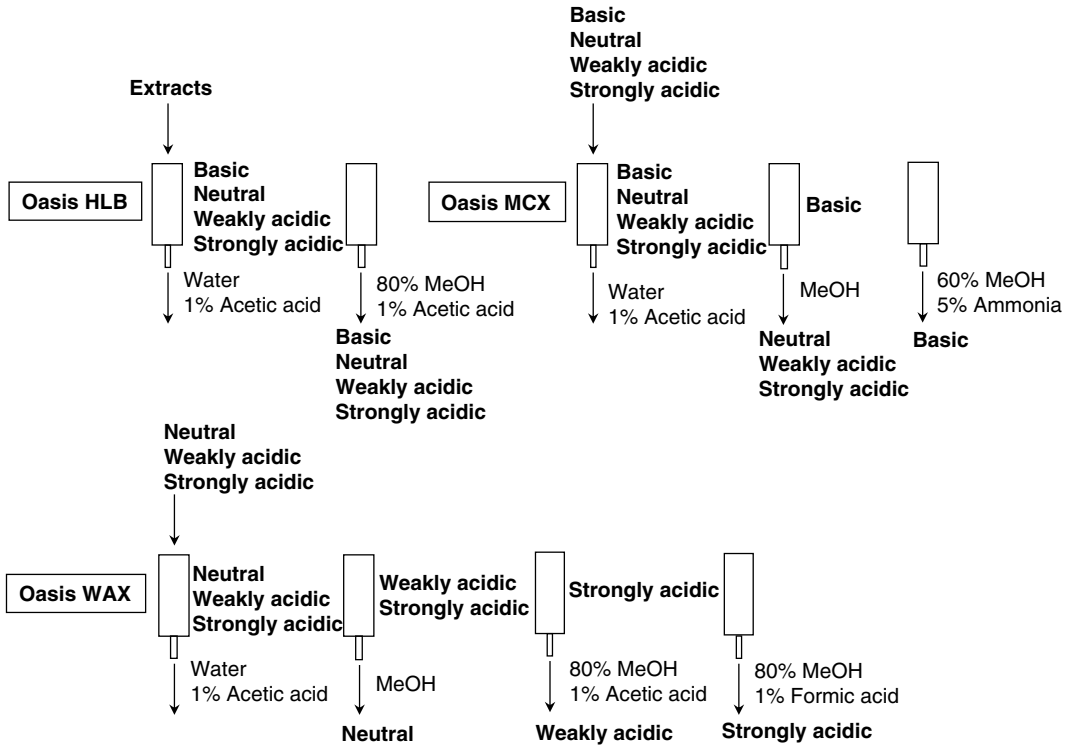

Fig. 2. Purification of the samples by column cartridge.

Extraction and partial purification are based on the procedure described in ref. (23) (Fig. 2). Ethylene is a gas and, thus, not suitable for this protocol. We have not succeeded in the quantification of BRs from relatively small amounts of plant materials (~1 g fresh weight vegetative tissues from *Arabidopsis*) by LC–ESI–MS/MS probably due to their lower abundance and occurrence of ion suppression during detection at MS/MS (see Note 2) (24). Relatively lower ionization efficiencies of BRs could be improved by derivatization (25). The method described here could be theoretically applied for most of the other hormone-related metabolites. Detailed methods for the quantification of ABA metabolites and GA metabolites are described in ref. (26) and (27), respectively (see Note 3).

Tof has a higher resolution compared to Q. Thus, we use the Q-Tof system for plant materials analyzed for the first time (plant species, tissue type, etc.) to be sure that there is no impurity in the detected ions. For example, a peak of the product ion for ABA (m/z 153) is detected from 153.09 to 153.1 m/z by Q-Tof, whereas the peak is detected from m/z 152.4 to 153.6 by Q-Q. This means that Q-Tof, but not Q-Q, can discriminate an impurity at m/z 153.0. On the other hand, Q-Q is generally more sensitive than Q-Tof and, thus, suitable for the quantification of hormones

and hormone metabolites in small-quantity samples. For accuracy, we confirm the consistency of the data using both systems at the beginning.

3.1. Sample Extraction and Purification

1. Prepare plant materials as required (see Note 4, Table 1). Freeze the materials in liquid nitrogen and store at −80°C until use.
2. Grind the plant materials into powder and add a certain volume of 80% MeOH containing 1% acetic acid as an extraction solvent (approximately 10 volumes of the sample fresh weight). Add internal standards (see Note 4) and extract at 4°C once for 1 h and then once for 10 min with additional extraction solvent.
3. Centrifuge at 14,000 × *g* for 10 min at 4°C. Collect supernatant and evaporate MeOH to obtain extracts in water containing acetic acid (see Note 5).
4. For desaltation, apply the extracts to an Oasis HLB column cartridge (see Subheading 3.2 for equilibration). After washing the cartridge with water containing 1% acetic acid to remove salts and high polar compounds, elute the plant hormones by 80% MeOH containing 1% acetic acid. Evaporate MeOH in the eluant to obtain extracts in water containing acetic acid (see Note 5).
5. Apply the extracts to an Oasis MCX column cartridge and wash the cartridge with water containing 1% acetic acid.
6. Elute the acidic (ABA, GAs, IAA, SA, JA) and neutral (BRs) hormones with MeOH.
7. Wash the cartridge with water containing 5% aqueous ammonia.
8. Elute basic hormones (tZ, DHZ, iP) with 60% MeOH containing 5% aqueous ammonia. Dry up the eluant and dissolve in water containing 1% acetic acid to inject into LC–ESI–MS/MS.

Table 1
Endogenous levels of hormones in *Arabidopsis* dry and imbibed seeds and the amount of internal standards used for the quantification from 50 mg dry seeds

		ABA	GA_1	GA_4	IAA	JA	SA	tZ	DHZ	iP
Dry seeds	Endogenous level (ng/g dry seeds)	135	nd[a]	nd[a]	68	21	1,085	nd[a]	nd[a]	0.003
	Internal standard (ng)	5	0.025	0.025	5	1.5	50	0.0025	0.0025	0.005
Imbibed seeds (24 h)	Endogenous level (ng/g dry seeds)	10	nd[a]	1.0	47	2.0	35	nd[a]	nd[a]	0.05
	Internal standard (ng)	1	0.025	0.025	2.5	0.25	2	0.0025	0.0025	0.005

[a] *nd* not detected; see Note 4

9. Add water containing 1% acetic acid to the fraction containing acidic and neutral hormones from step 6 and evaporate MeOH (see Note 5).
10. Apply the fraction to an Oasis WAX column cartridge (see Subheading 3.2 for equilibration) and wash the cartridge with water containing 1% acetic acid.
11. Elute the neutral hormones (BRs) with MeOH.
12. Elute the weakly acidic hormones (ABA, GAs, IAA, JA) by 80% MeOH containing 1% acetic acid. Dry up the eluant and dissolve in water containing 1% acetic acid to inject into LC–ESI–MS/MS (see Note 5).
13. Elute the strongly acidic hormone (SA) by 80% MeOH containing 1% formic acid. Dry up the eluant and dissolve in water containing 1% formic acid to inject into LC–ESI–MS/MS.
14. Dry up the fraction containing the neutral hormones (BRs) from step 11 and then dissolve in chloroform.
15. Apply the extracts to a SepPak silica column cartridge (see Subheading 3.2 for equilibration) and wash with chloroform.
16. Elute the neutral hormones (BRs) with chloroform/MeOH (9:1 v/v). Dry up the eluant and dissolve in 50% MeOH to inject into LC–EIS–MS/MS.

3.2. Column Cartridge Equilibration

1. Oasis HLB: Wash with 1 volume of MeCN and then with MeOH. Equilibrate with 1 volume of initial solvent (water containing 1% acetic acid).
2. Oasis MCX: Wash with 1 volume of MeCN and then with MeOH. Regenerate with 0.5 volume of 0.1 M HCl. Equilibrate with 1 volume of initial solvent (water containing 1% acetic acid).
3. Oasis WAX: Wash with 1 volume of MeCN and then with MeOH. Regenerate with 0.5 volume of 0.1 M NaOH. Equilibrate with 1 volume of initial solvent (water containing 1% acetic acid).
4. SepPak silica: Wash and equilibrate with 3 volumes of chloroform.

3.3. LC–ESI–MS/MS Analysis

1. Set the LC conditions: Flow rate, 200 μl/min; typical gradients of two solvents are listed in Table 2 (see Note 6).
2. Set the MS/MS conditions: Q-Tof premier: capillary, 2.8 kV; source temperature, 80°C; desolvation temperature, 400°C; cone gas flow, 0 L/h; desolvation gas flow, 500 L/h. Agilent 6410: capillary, 4,000 V; desolvation temperature, 300°C; gas flow, 9 L/min; nebulizer, 30 psi. Typical MS/MS transitions, collision energy, and sampling voltage are summarized in the Tables 3 and 4 (see Note 6).

Table 2
LC conditions

Method no.	Solvent A	Solvent B	Gradient (composition of solvent B)
1	Water containing 0.01% acetic acid	MeCN, 0/05% acetic acid	3–50% over 20 min
2	Water containing 0.01% acetic acid	MeCN, 0/05% acetic acid	3–25% over 27 min
3	0.1% formic acid	MeCN, 0/1% formic acid	3–98% over 10 min

Table 3
Parameters for LC–ESI–MS/MS analysis (ACQUITY UPLC-Q-Tof premier)

	LC method	Retention time on LC (min)	ESI	MS/MS transitions for quantifications (*m/z*)	Collision energy (V)	Sampling cone voltage (V)
ABA D_6-ABA	1	8.5	–	263/153 269/159	8	22
GA1 D_2-GA1	1	5.7	–	347/273 349/275	20	40
GA4 D_2-GA4	1	11.8	–	331/257 333/259	20	40
IAA D_2-IAA	1	7.3	+	176/130 178/132	10	16
JA D_2-JA	1	10.1	–	209/59 211/59	8	20
SA D_6-SA	3	4.2	–	137/93 141/97[a]	12	25
tZ D_6-tZ	2	5.8	+	220/136 225/136, 137	16	30
DHZ D_3-DHZ	2	6.1	+	222/136 225/136	20	35
iP D_6-iP	2	14.8	+	204/136 210/137	12	25

[a]Deuterium atoms at the hydroxy and carboxyl groups are immediately exchanged with hydrogen atoms in water-containing solutions

3. Determine the amount of each compound by spectrometer software (MassLynx™ v. 4.1 or MassHunter™ v. B. 01. 02). Typical MS chromatograms obtained by the ACQUITY UPLC-Q-Tof premier system are presented in Fig. 3.

Table 4
Parameters for LC–ESI–MS/MS analysis (Agilent 1200-6430)

	LC method	Retention time on LC (min)	ESI	MS/MS transitions for quantifications (*m/z*)	Collision energy (V)	Fragmentor (V)
ABA D_6-ABA	1	10.8	–	263/153 269/159	8	140
GA1 D_2-GA1	1	8.0	–	347/273 349/275	24	150
GA4 D_2-GA4	1	14.1	–	331/257 333/259	26	150
IAA D_2-IAA	1	9.9	+	176/130 178/132	18	110
JA D_2-JA	1	12.6	–	209/59 211/59	10	150
SA D_6-SA	3	6.1	–	137/93 141/97[a]	16	100
tZ D_6-tZ	2	7.8	+	220/136 225/136, 137	16	110
DHZ D_3-DHZ	2	8.1	+	222/136 225/136	20	110
iP D_6-iP	2	17.4	+	204/136 210/137	14	100

[a]Deuterium atoms at the hydroxy and carboxyl groups are immediately exchanged with hydrogen atoms in water-containing solutions

4. Notes

1. We use the columns with small particle size (1.7 μm for ACQUITY UPLC BEH C18 and 1.8 μm for ZORBAX Eclipse XDB-C18). Theoretically, these columns give approximately three times higher resolution compared to the popularly used columns with 5-μm particles. However, injection of relatively large volume of samples results in broader peaks.
2. Ionization of the target molecules by ESI is inhibited if a large amount of impurity exists in the same retention time on LC due to the absorption of charged molecules by impurity. As a result, the targets cannot be introduced efficiently to MS/MS, resulting in reduced detection or loss of detection. This phenomenon is called “ion suppression” and is a demerit of LC–ESI–MS/MS. Even if a certain amount of a pure standard compound is detectable, it does not mean that the amount

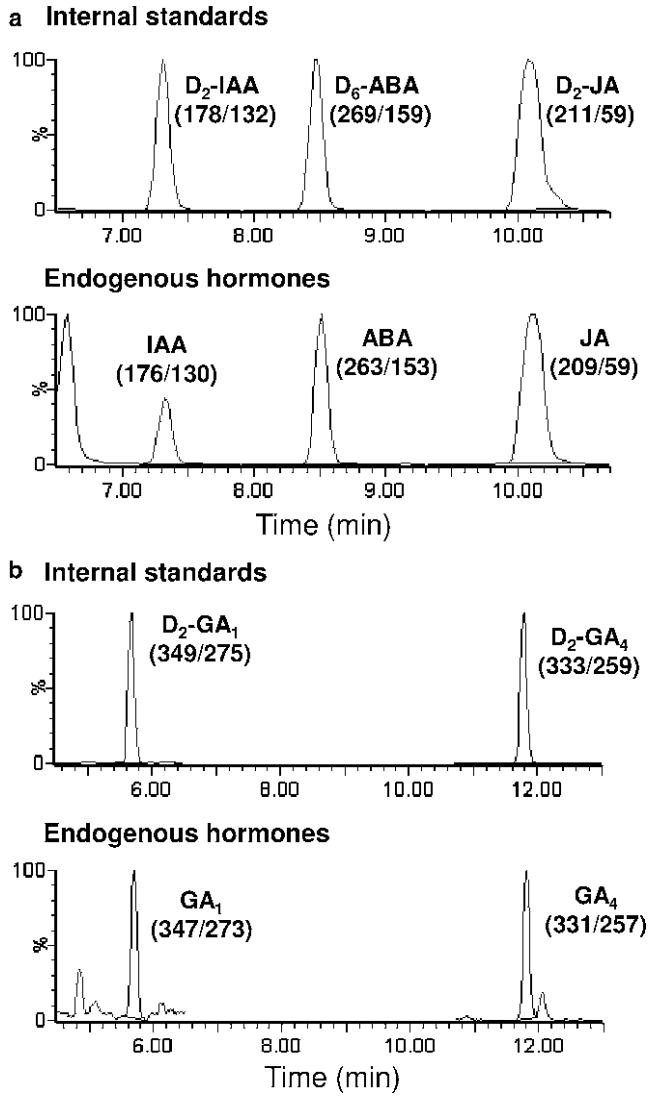

Fig. 3. MS chromatograms for weakly acidic hormones obtained by ACQUITY UPLC-Q-Tof premier. (**a**) MS chromatograms for IAA, ABA, and JA extracted from 50 mg *Arabidopsis* dry seeds. Endogenous GA_1 and GA_4 were not detected in this condition. (**b**) MS chromatograms for GA_1 and GA_4 extracted from 100 mg (fresh weight) *Arabidopsis* flowers.

is sufficient to detect the given compound in plant materials. Additional purification steps (see Note 7) are required if the effect of ion suppression is not negligible.

3. Purification steps are basically the same, but additional purification is required depending on the quantity and quality of the starting materials. Conditions for LC (Tables 1 and 2) have to be modified for separate metabolites that have similar chemical properties.

4. The amount of starting plant materials depends on the target compounds to be analyzed and on the type (species, tissues, and physiological status) of the materials. Examples of analyzed hormone levels in wild-type *Arabidopsis* (Col-0) dry seeds and imbibed seeds measured by the ACQUITY UPLC-Q-Tof premier system are presented in the Table 1. Internal standards with stable isotope labels are added to the samples at the beginning of extraction, and endogenous hormone levels are calculated based on the relative ratio to the internal standards. The amount to be added to the extracts is estimated from the endogenous levels of the targets (Table 1). Endogenous levels of hormones vary between samples. For example, ABA levels in freshly harvested dry seeds range from c.a. 50 to 150 ng/g dry weight. Endogenous GA_4, which is the major form of bioactive GA in *Arabidopsis*, is often not detectable from 50 mg of dry seeds as shown in Table 1, but sometimes is detectable. Samples must be prepared from plants grown in the same conditions at the same time. It is also important to analyze biological replicates with different batches.
5. Fractions that contain volatile compounds, such as JA, should not be kept under negative pressure conditions for a long time after they are completely dried.
6. The conditions presented in Tables 2–4 are examples in our experimental conditions. Settings have to be optimized for each system.
7. If required, for example, when relatively large amounts of plant materials are analyzed, additional purification steps could be introduced prior to HLB column purification as follows: partition the MeOH extracts against *n*-hexane and discard hydrophobic compounds in the *n*-hexane phase. Evaporate MeOH, resuspend the pellet in phosphate buffer (pH 8.0), and apply to the column containing PVP to remove polyphenols. PVP columns are not commercially available. Extract with ethyl acetate to obtain the acidic and neutral fractions. Extract with chloroform to obtain the basic fraction.

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Chapter 8

In Vitro Assay for ABA 8′-Hydroxylase: Implications for Improved Assays for Cytochrome P450 Enzymes

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Abstract

In vitro assays for cytochrome P450 enzymes developed from plant-derived microsomal extracts have not been used extensively for the characterization and quantification of enzyme activities in plant tissues. We describe here an in vitro assay for abscisic acid (ABA) 8′-hydroxylase that was developed using microsomes extracted from (+)-ABA-induced corn suspension cultures. This assay may be useful for further characterization and monitoring of ABA 8′-hydroxylase activities in germinating seeds, seedlings, and other tissues. Additionally, the optimization protocols provided here may be adapted towards improving in vitro enzyme assays for other cytochrome P450 enzymes expressed in plants.

Key words: Cytochrome P450, In vitro assay, Microsomes, (+)-ABA 8′-hydroxylase, Abscisic acid, Phaseic acid, Germination, Dormancy

1. Introduction

Cytochrome P450 enzymes are responsible for a wide array of biological oxidations in plants, including the production of secondary metabolites, primary structural compounds, and several plant hormones (e.g., auxins, gibberellins, brassinosteroids, abscisic acid (ABA)) (1–3). In particular, a large number of distinct cytochrome P450 enzymes are involved in upstream pathways for the synthesis of phytohormone precursors, late conversions of these to more active forms, and subsequent catabolism of phytohormones to less active (or inactive) metabolites (1–3).

Four members of the CYP707A subfamily of cytochrome P450 enzymes in *Arabidopsis* (i.e., CYP707A1, CYP707A2, CYP707A3, CYP707A4) are responsible for the oxidative inactivation of ABA to its biologically less active metabolite, 8′-hydroxyABA (4–7). This latter compound cyclizes spontaneously to the stable and

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(almost) inactive metabolite, phaseic acid (PA), and as a consequence, the effective levels of the active hormone are dramatically reduced by these tandem reactions. The CYP707A subfamily genes are expressed at high levels in imbibing and germinating seeds, young seedlings, leaves/stomata, and in response to stress or leaf/stem submergence (6–10). Most importantly, the enzyme activity associated with this subfamily (e.g., CYP707A2) has been implicated in the catabolism/inactivation of ABA in seeds to promote seed germination and overcome dormancy (11–16).

In many cases, it has been difficult to measure cytochrome P450 activities directly in extracts from plant tissues. Characteristically, these enzymes are in low abundance and enzyme activity measurements are inferred by indirect means from gene expression measurements based on northern blots, PCR-based assays, EST frequencies, or microarray data. A robust in vitro assay developed from plant microsomal extracts can provide direct quantitative and qualitative data for a particular enzyme activity across tissues and developmental stages. We present here a detailed protocol for the development and optimization of the in vitro assay for ABA 8′-hydroxylase (4) and suggest where it might be applied to future studies of germination and dormancy, and as a model for studies of cytochrome P450 enzymes in general. With recent improvements in the sensitivity of detection methods for various metabolites, it may now be possible to analyze and measure the in vivo activities of highly expressed cytochrome P450s through tissue-based in vitro assays on a more routine basis (17–20).

2. Materials

2.1. Corn Suspension Culture and Tobacco Seedlings

1. Black Mexican Sweet (BMS) corn suspension cultures (*Zea mays* L. cv. Black Mexican Sweet) are initiated and maintained as described previously (21). A culture was grown continuously at the NRC-Plant Biotechnology Institute (PBI) under sterile conditions for more than 20 years. Although these cells are no longer available from NRC-PBI, verified cultures (callus or suspension) are currently available from the German Collection of Microorganisms and Cell Cultures (DSMZ; http://www.dsmz.de/plant_cell_lines/; cat. PC-1116).
2. Thiamine stock solution: 0.5 mg/mL. Use tissue culture quality water (see Note 1) and store at 4°C protected from light.
3. 2,4-D stock solution: 0.5 mg/mL. The 2,4-D (Sigma-Aldrich) is first dissolved in ethanol, and then water is added to make the correct volume. Use tissue culture quality water (see Note 1) and store at 4°C protected from light.
4. Culture medium for the BMS corn suspension cultures (SPECIAL2-MS Medium; SP2-MS): This is prepared from

single-strength (1×; 4.4 g/L) Murashige and Skoog (MS) Basal Medium (22) (Sigma-Aldrich; M5519). The MS basal medium contains macronutrients, micronutrients, and vitamins and is supplemented with 0.5 mg/L thiamine (1 mL of 0.5 mg/mL thiamine stock per L of medium), 2 mg/L 2,4-D (4 mL of 0.5 mg/mL 2,4-D stock per L of medium), 150 mg/L L-asparagine, and 20 g/L sucrose with pH adjusted to 5.8.

5. Aliquots of the SP2-MS medium are placed into 250-mL Erlenmeyer flasks (50 mL in each). These are stoppered with cotton ball plugs (rolled cotton batting with a cheesecloth outer layer) and autoclaved for 30 min.
6. Vacuum apparatus: Büchner funnel (with rubber bung adapter) set on a large Büchner flask (side-arm flask) (Fisher Scientific) with low vacuum applied through a water aspirator. If necessary, the flask is securely clamped to prevent it from falling over.
7. ABA stock solution for induction of corn suspension cultures: 25 mg of (+)-ABA (Sichuan Lomon Corporation, Sichuan, China; see Note 2) is dissolved in 1.25 mL of 100% ethanol to make a 76 mM stock solution (F.W. 264.3 g) (see Note 3). The stock solution is tightly sealed, protected from light, and stored at −20°C. Amber vials with a lined cap are useful for storing ABA and ABA metabolite stock solutions.
8. Ethanol (100%), High-performance liquid chromatography (HPLC) grade.
9. Whatman No. 1 filter paper, sized to fit the Büchner funnel.
10. Aluminum foil.
11. Spatula (Spoonula Lab Spoon; Fisher Scientific).
12. Tobacco: *Nicotiana tabacum* L cv. Xanthi NN (Lehle Seeds, Round Rock, TX). Tobacco seedlings are grown at 22°C, 16/8 h light/dark cycle. Cotyledons and expanding leaves are harvested from 9- and 15-day-old seedlings by shearing the seedlings at the surface of the soil (roots are discarded). This tissue is frozen, stored at −80°C, extracted, and analyzed for ABA 8′-hydroxylase activity as described for corn suspension cells.

2.2. Microsomal Fraction Preparation

1. A mortar and pestle (8.8-cm O.D. mortar) provides the best grinding action with these volumes of tissue.
2. 200 mM stocks of potassium phosphate monobasic (KH_2PO_4) and potassium phosphate dibasic (K_2HPO_4). Do not adjust the pH. To make a 200 mM potassium phosphate buffer (pH 7.6), mix together prescribed volumes of the 200 mM monobasic and dibasic potassium phosphate solutions with stirring and monitoring using a pH meter, until pH 7.6 is reached (requires volumes in the ratio of ~15:85, monobasic to dibasic). Store at 4°C.

3. Extraction buffer: 0.1% bovine serum albumin (BSA), 0.33 M sucrose, 40 mM ascorbate, 100 mM K-phosphate buffer pH 7.6 (from stock above) (see Note 4). Make fresh each time and chill on ice.
4. Miracloth (EMD Chemicals, Gibbstown, NJ).
5. Weighing boat: Hexagonal polystyrene weighing dish (bottom I.D. 2.5 cm, top I.D. 3.6 cm) (Fisher Scientific).
6. Corex centrifuge tubes (15 mL; now manufactured by Kimble) (see Note 5). Adaptors are required for rotors accommodating 50-mL tubes.
7. High-speed refrigerated preparative centrifuge: Sorvall RC-5B with SS-34 fixed-angle rotor (8 × 50 mL) and adaptors or an equivalent centrifuge and rotor.
8. Polyethylene transfer pipettes (various sizes): Large opening (and nonbreakable) for resuspending pellets and transferring supernatants (Fisher Scientific).
9. Ultracentrifuge: Beckman L8-M with TY-65 fixed-angle rotor and Beckman polyallomer Quick-Seal tubes (16 × 76 mm; capacity 12.5 mL) (Beckman Coulter, Brea, CA). Use tubes recommended by the manufacturer for that rotor. The Quick-Seal tubes may require a sealing device. Proper operation of an ultracentrifuge requires strict adherence to the manufacturer's instructions and these should be reviewed and consulted at all stages.
10. Resuspension buffer (same as assay buffer, see below): 100 mM K-phosphate pH 7.6 or 100 mM NaOH-HEPES pH 7.6.

2.3. In Vitro ABA 8′-Hydroxylase Assay

1. Screw-capped 1.5-mL conical microfuge tubes with O-ring.
2. 1 N HCl (diluted with water from concentrate).
3. Bio-Rad Protein assay (Bio-Rad) (23).
4. Glass test tubes (13 × 100 mm).
5. Protein standards (BSA; Sigma-Aldrich): These are prepared from a 20 mg/mL stock and stored in 1-mL aliquots at –20°C. This concentrated solution is thawed, mixed thoroughly, diluted to 2 mg/mL with resuspension buffer, and then further diluted with measured aliquots of resuspension buffer in 1.5-mL microfuge tubes to give a set of eight BSA standard solutions (1 mL each) at concentrations ranging from 100 to 1,400 μg/mL (see Note 6).
6. Spectrophotometer: Visible range required. Use disposable polystyrene cuvettes (Bio-Rad).
7. Assay buffer (same as resuspension buffer): 100 mM K-phosphate pH 7.6 or HEPES-NaOH, pH 7.6. Use either the K-phosphate buffer for both resuspension of the microsomal

pellet and the in vitro assay or use the HEPES-NaOH buffer for the resuspension and assay, but do not include both buffers in the same experiment. We used the K-phosphate buffer almost exclusively.

8. NADPH (Sigma-Aldrich). Make a fresh quantity of 10 mM NADPH in resuspension buffer each time. Keep on ice. Use the highest quality of NADPH that is available.
9. Radioactive substrate for the in vitro assay: [^{3}H]-(+)-ABA. This was prepared for us by others and is reasonably stable under proper storage conditions (Specific Activity: from 30 to 300 Ci/mole, depending on the lot). The [^{3}H]-(+)-ABA is dissolved in 100% ethanol, measured aliquots are distributed to several dozen preweighed vials, solvent is evaporated from each vial, and these aliquots of [^{3}H]-(+)-ABA are stored at −20°C (sealed and protected from light) for long-term storage. When required, stocks are made by dissolving the [^{3}H]-(+)-ABA residue in each vial in 100% ethanol to a final concentration of 20 mM (up to 6.86 μCi/μL); these solutions are stored at −20°C (tightly sealed and protected from light). One microliter of the 20 mM [^{3}H]-(+)-ABA stock and 4 μL of 20 mM nonradioactive (+)-ABA (see below) are added to each assay reaction tube (200 μM final concentration) to initiate the enzyme reaction. Anyone wishing to pursue this detection method should obtain the radiolabeled material by contract from an institute having the appropriate radiation license and chemistry expertise (see Note 7). Observe all stipulated precautions when handling radioactive material, and dispose of all waste properly.
10. Nonradioactive substrate for the in vitro assay: (+)-ABA is dissolved in 50 mM sodium carbonate at 20 mM. Store at −20°C (seal tightly and protect from light). For nonradioactive assays, 5 μL of the 20 mM stock is added to each assay reaction tube for a final concentration of 200 μM (see Note 2).
11. Thermomixer (Eppendorf) (see Note 8).

2.4. Detection and Quantification of ABA 8′-Hydroxylase Activity

1. Glass screw-cap round-bottomed test tubes with lined caps (Fisher Scientific; 13 × 100 mm).
2. Solvents: High-quality (or HPLC grade) methanol, ethanol, ethyl acetate, toluene, acetic acid, and acetonitrile.
3. Nitrogen tank with valve and evaporating apparatus: To increase the evaporation of the ethyl acetate, the tubes are placed in a 24 place heating block (use very low heat) and a manifold with appropriately placed blunt needles is positioned above the tubes to gently dispense the nitrogen gas. Use in a fume hood.
4. Waters Oasis HLB extraction cartridges (Waters, Milford, MA).

5. Hamilton Gastight syringes with blunt needle tip, 50 and 10 µL (Fisher Scientific).
6. (+)-ABA standard (nonradioactive) for thin-layer chromatography (TLC). Dissolve (+)-ABA at 3 mg/mL in 100% ethanol; use 6 µL for each spot required on the TLC plate. Store solution at −20°C (seal tightly and protect from light). The other ABA-related standards are prepared similarly.
7. ABA and ABA metabolites used as standards for TLC and HPLC detection were purchased commercially (Sigma-Aldrich) or synthesized in-house (NRC-PBI). Some ABA metabolites can be purchased from the NRC-PBI (http://www.nrc-cnrc.gc.ca/eng/facilities/pbi/plant-hormone.html).
8. TLC plates: Silica gel aluminum-backed plates with a fluorescence indicator, 20 × 20 cm (EMD Chemicals).
9. TLC solvent system: Toluene:ethyl acetate:acetic acid (25:15:2, v/v).
10. Ethanol.
11. Chromatography tanks with lids.
12. Whatman chromatography paper (Grade 3).
13. Handheld UV light and eye protection.
14. EN^{3}HANCE spray surface autoradiography enhancer (Perkin Elmer, Waltham, MA).
15. Plastic food wrap.
16. X-ray film: Kodak X-OMAT XRP-1 or BioMax XAR film (8 × 10 in.; Perkin Elmer).
17. Kodak Biomax Film Cassette without screen (Perkin Elmer).
18. HPLC system.
19. Supelcosil HISEP reverse-phase column (150 × 4.6 mm).
20. HPLC solvent system: 75:25 (v/v) mixture of 1% acetic acid (in water) and acetonitrile.
21. Sample vials and capping tool for HPLC analysis.

3. Methods

Cytochrome P450 enzymes are typically associated with the endoplasmic reticulum (ER), so successful development of an authentic in vitro assay requires preparation of a microsomal fraction enriched in ER by centrifugation. However, successful assays are contingent upon identification of a tissue that displays or can be induced to display high expression of the enzyme activity in question. Isolation of the microsomal fraction must be done in such a manner as to

preserve the activity, and in vitro assay conditions must support optimal activity. Our studies of the ABA 8′-hydroxylase enzyme led us to develop an in vitro assay using both radioactive and nonradioactive substrates, which could be used for studies of enzymatic parameters and classification of agonists and inhibitory substrate analogs (5). As well, such studies could be used for quantifying kinetic parameters in vitro and improving an understanding of metabolic flux in vivo.

Using these techniques, an in vitro assay for ABA 8′-hydroxylase activity was developed for (+)-ABA-induced BMS corn suspension cultures, and later for germinating seedlings of tobacco to document natural plant levels of this enzyme activity. In germinating tobacco seedlings, the enzyme activity was estimated at approximately 1,000 times lower than in membranes isolated from the (+)-ABA-induced corn suspension cell cultures. The amount of ABA 8′-hydroxylase activity measured in microsomes from tobacco seedlings is similar to the values reported previously for this enzyme from embryonic axes of chickpea (24).

3.1. BMS Corn Suspension Cultures and Induction of ABA 8′-Hydroxylase

1. BMS corn suspension cultures are grown in liquid SP2-MS Medium (MS Basal Medium supplemented with 0.5 mg/L thiamine, 2 mg/L 2,4-D, 150 mg/L l-asparagine, and 20 g/L sucrose) at room temperature with gentle shaking (100 rpm) and diffuse fluorescent light (day length in the lab may be variable) (21).These are subcultured twice a week (Mondays and Fridays), with transfer of half of the culture (approximately 50 mL) into 50 mL of fresh media in a 250-mL flask. To maintain sterility, flasks are stoppered with tight-fitting cotton ball plugs made from rolled cotton batting and a layer of cheesecloth. Additional long-term cultures are maintained on solid SP2-MS media in Petri plates, sealed with parafilm, and incubated at room temperature under low light (in a plastic box); these require subculturing once a month.
2. (+)-ABA 8′-hydroxylase activity is induced in fresh BMS corn suspension cultures (24 h after subculturing, see Note 9) with addition of approximately 200 μM (+)-ABA (250 μL of 76 mM stock). The cultures are incubated in the dark for 16 h with gentle shaking (100 rpm).
3. After 16 h of incubation with (+)-ABA, cells are harvested by gentle vacuum filtration to remove excess media. Cultures are slowly poured onto two layers of dampened Whatman No. 1 filter paper in a vacuum apparatus. The collected cells are immediately weighed in 2.5 g aliquots in small polystyrene weighing boats, wrapped securely in aluminum foil (includes the weighing boat), and dropped into liquid nitrogen for rapid freezing. These premeasured aliquots of cells (2.5 g) are stored at −80°C until required. Sterile conditions are not required during harvest.

3.2. Extraction of Microsomes for In Vitro Assay

1. Wear laboratory quality gloves for all subsequent steps in all sections of all protocols.
2. The preparative centrifuge (Sorvall RC-5B), tube adaptors, and rotor (SS-34 fixed-angle rotor) are prechilled to 4°C.
3. Frozen tissue (2.5 g) is ground rapidly to a powder with added liquid nitrogen in a prechilled mortar with pestle (mortar and pestle chilled to –20°C) (see Note 10).
4. Eight volumes (20 mL) of extraction buffer at 4°C are added as the last of the liquid nitrogen is vaporized, and gently mixed with the frozen ground tissue using the pestle (see Note 11). The mortar is kept on ice during this step to keep the tissue extract as cold as possible.
5. The tissue homogenate is filtered through two layers of Miracloth into a chilled 50-mL beaker on ice, and then divided between two 15-mL Corex centrifuge tubes (prechilled on ice). Tubes in adaptors occupying opposite positions in the rotor are adjusted to identical weights (see Note 5).
6. The samples are centrifuged at 13,100 × *g* (12,000 rpm) for 15 min at 4°C in a Sorvall RC-5B preparative centrifuge using an SS-34 rotor with adaptors (see Note 5). After centrifugation, the supernatants are removed with a pipette and collected in cold beakers on ice (identical treatments are pooled). The pelleted cellular debris is discarded (see Note 12).
7. The collected supernatants are recentrifuged at 200,000 × *g* (55,000 rpm) under vacuum at 4°C for 60 min in sealed and balanced ultracentrifuge tubes in a high-speed ultracentrifuge. We used a Beckman L8-M ultracentrifuge with a fixed-angle TY 65 rotor and polyallomer Quick-Seal tubes. Operation of an ultracentrifuge requires strict adherence to the manufacturer's instructions and these should be reviewed and consulted at all steps. Follow the manufacturer's recommendations for choice of rotor and tube for your ultracentrifuge. Always accurately weigh oppositely placed tubes (and adjust weights if necessary) to properly balance the rotor, and practice sealing tubes before using your protein sample. After centrifugation, the tubes are opened, the supernatant is discarded, and the pelleted membrane fraction is retained.
8. Each microsomal pellet is resuspended (on ice) with 0.625 mL of cold (4°C) resuspension buffer (1.25 mL/original 2.5 g tissue) by gentle aspiration with a plastic disposable pipette. The enzyme solution is stored on ice until required (see Note 13).
9. Protein concentration of the resuspended pellet is measured using the BioRad protein assay (BioRad) with BSA standards from 100 μg/mL to 1,400 μg/mL (see Note 14). One hundred microliters of each solution (sample or protein standard) is pipetted into a clean glass test tube. Add 5.0 mL of the

diluted dye reagent (see manufacturer's instructions) to each tube and vortex to mix thoroughly. Allow the assay to develop for 30 min. Measure the absorbance at 595 nm against a blank consisting of 100 µL of the resuspension buffer and 5.0 mL of dye reagent, incubated similarly to the protein-containing samples. The microsomal sample(s) might have to be diluted to get an accurate measure of protein concentration.

10. Alternative methods for preparing microsomes, involving Ca^{2+} or polyethylene glycol (PEG) precipitation of membranes and low-speed centrifugation, also have produced satisfactory results (25, 26) and may be used when some equipment (i.e., ultracentrifuge) is not available.

3.3. In Vitro Assay for ABA 8′-Hydroxylase

1. A sensitive, but poorly quantitative, measure of enzyme activity was developed first using radiolabeled [^{3}H]-(+)ABA as the precursor substrate. This first in vitro assay utilized a radioactive substrate, [^{3}H]-(+)ABA, followed by ethyl acetate extraction, separation of metabolites by TLC, and fluorography to visualize the metabolites (Fig. 1a).
2. When using radioactive materials, follow all guidelines for using such materials to prevent contamination of lab spaces and equipment. The enzyme assays with radioactive substrates are conducted in screw-capped conical 1.5-mL microfuge tubes fitted with O-rings to prevent any leakage and/or aspiration of the radioactive materials into the centrifuge rotor and casing.
3. The hydroxylation reaction is initiated by the addition of 5 µL of substrate (1 µL of [^{3}H]- (+)-ABA and 4 µL of nonradioactive (+)-ABA from 20 mM stocks) to a 1.5-mL microfuge tube containing an aliquot of microsomal suspension (0.3–0.6 mg protein), diluted with assay buffer to 245 µL, and 250 µL of NADPH (from 10 mM stock; freshly prepared in assay buffer). The total reaction volume after the substrate is added is 500 µL.
4. Mix gently, but do not vortex the assay mixture.
5. The NADPH concentration for optimal activity is 5 mM.
6. When developing an in vitro assay for any enzyme, it is necessary to include several controls (for example, inactivated protein preparation (boiled) versus active preparation, plus and minus cofactor (NADPH)) in order to ensure that the activity detected (i.e., product accumulated) is genuinely due to the expected enzyme reaction (Fig. 1a).
7. The reaction is incubated for 3 h at 30°C with gentle shaking in a temperature-controlled incubator (Thermomixer) (see Note 15). The reaction is stopped by the addition of 0.2 volumes of 1 N HCl to each tube and centrifuged for 5 min to

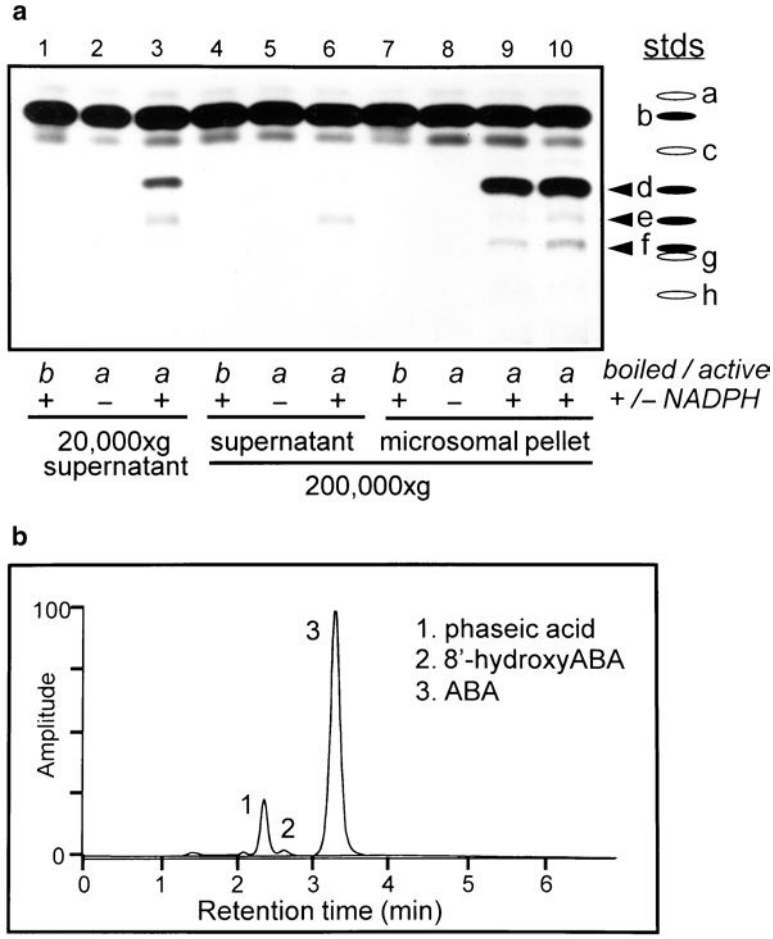

Fig. 1. (+)-ABA 8′-hydroxylase activity in microsomes extracted from BMS corn suspension cultures. (**a**) After tissue homogenization, the initial 20,000 × *g* supernatant and subsequent 200,000 × *g* supernatant and microsomal pellet were assayed for in vitro ABA 8′-hydroxylase activity with [^{3}H]-(+)-ABA as a substrate. The pellet was resuspended in 0.1 volume of the original homogenate, and therefore proteins were concentrated tenfold over the first supernatant or the original homogenate. Identification of substrate and products on TLC plates was based on comigration with nonradioactive authentic standards (identified as fluorescent spots under UV light) that had been run in separate lanes or superimposed on the assay extracts for verification of comigration. Standards (stds) used were: a, *trans*-ABA; b, ABA; c, (±)-ABA *trans*-1′,4′-diol; d, phaseic acid (PA); e, (±)-ABA *cis*-1′,4′-diol; f, 8′-hydroxyABA; g, 7′-hydroxyABA; and h, dihydrophaseic acid (DPA). The *filled arrowheads* indicate the ABA metabolites identified in these assays. (**b**) Output from an HPLC separation using a reverse-phase column with UV detection at 262 nm. (Reproduced from ref. 4).

remove precipitated protein (high speed at room temperature in a microcentrifuge).

8. Ethyl acetate extraction: This entire extraction must be done in a fume hood. The assay samples are transferred to individual glass screw-top round-bottomed test tubes (13 × 100 mm) with lined caps to prevent solvent spillage and allow for vigorous vortexing. ABA and ABA metabolites are isolated from the acidified and clarified assay mixture by repeated extraction (3×) with 3 mL of ethyl acetate each time and vortexing for 30 s

(see Note 16). After vortexing, the ethyl acetate/aqueous mixture is allowed to separate into two phases by standing or low-speed centrifugation in a bench-top swing bucket centrifuge. The upper phase (ethyl acetate phase), now containing ABA and its metabolites, is collected into a clean glass test tube using a glass Pasteur pipette. After successive extractions of the aqueous phase, the collected ethyl acetate fractions are evaporated under a stream of nitrogen gas with gentle warming in the fume hood. The dried residue (containing ABA and ABA metabolites) is redissolved in 100 μL of methanol for sample analysis by TLC (see Note 17).

9. TLC separation: Extracts of in vitro assays using [^{3}H]-(+)ABA as substrate are separated on TLC plates (with a fluorescence indicator). Note that all chromatography steps should be conducted in a fume hood.
10. The TLC plate is prepared by lightly drawing a pencil line (using a soft pencil) evenly about 2.5 cm above the bottom edge. This line is placed such that samples spotted along this line are entirely above the initial solvent front when the plate is placed within the chromatography tank. Pencil dots are placed equidistant along this line (1.5 cm apart) to show the positions where the individual samples are applied.
11. A measured aliquot of the methanol extract for each of the samples (20–40 μL) is carefully spotted in small increments at one of the pencil dots on the TLC plate (with drying between each application to concentrate the material) using a 50-μL Hamilton Gastight glass syringe. Additional samples are applied successively to the plate and allowed to dry completely before chromatography in a sealed chromatography tank in a fume hood.
12. All chromatography steps are conducted in a fume hood. Initially, the plate is run for only 4–5 min with a small amount of ethanol in a chromatography tank such that the initial solvent level in the tank is below the sample positions on the TLC plate. This short pre-run vertically concentrates the samples at a new origin.
13. After drying, the plate is run in the TLC chromatography solvent (toluene:ethyl acetate:acetic acid; 25:15:2, v/v). This tank is set up prior to spotting the plate to ensure an equilibrated and saturated atmosphere. A piece of chromatography paper placed along the back wall of the tank acts as a wick to improve equilibration (see Note 18).
14. The chromatography run is allowed to proceed until the solvent front is at a predetermined point, ~2.5 cm from the upper edge of the plate (this takes approximately 1 h). The TLC plate is removed and dried in the fume hood. The plate is run a

second time in fresh solvent, in a second tank, equilibrated as before. ABA has an R_f of approximately 0.50 at the completion of the chromatography runs.

15. After air drying, the plate is examined using a handheld UV light. The fluorescing standards and samples are marked (circled with a pencil) on the TLC plate. Eye protection (i.e., UVEX glasses) must be worn while using the UV light.
16. The TLC plate is sprayed with the EN^{3}HANCE autoradiography enhancer in a fume hood, dried for 30 min in a fume hood, and then wrapped securely in plastic food wrap (e.g., Saran Wrap). The wrapped TLC plate is placed onto film in a darkroom. All manipulations with the film occur in a light tight darkroom with a red (film-safe) light to allow viewing. It is very important to align and record the position of the TLC plate against the X-ray film to confirm the relative positions of the samples, substrate and metabolites for later. We mark the upper right corner of the X-ray film by a diagonal cut and tape the wrapped TLC plate to the film, marking that position with a permanent marker. The film is placed in a film cassette, and this is further wrapped in light tight material (foil or a photography bag) and placed in a −80°C freezer for 24 h. The film is developed using an automatic film developer or using standard X-ray film development solutions available from Kodak.
17. After the film is developed, comparisons are made between the marked positions of the metabolites and standards on the TLC plate (previously viewed under UV light) and the positions of the radioactive compounds visualized on the film. The identification of substrate and products is made by comparisons to the migration patterns of authentic standards applied in additional lanes. Identifications can be confirmed by comigration of the radioactive metabolites from the in vitro assays with separately spotted quantities of unlabeled standards. In comigration comparisons, the radioactive experimental sample is spotted onto the TLC plate first, and after drying, the unlabeled standard for comparison also is spotted on the same place on the TLC plate.
18. The radioactive assay was the most sensitive assay and permitted the initial optimization of the in vitro assay (Fig. 1). Thereafter, it was most convenient and efficient to switch to a nonradioactive assay with detection of the products by HPLC. This permits the quantitative measurement of enzyme activities and broader assessments of activity-related parameters within a single experiment (Fig. 1b). The nonradioactive assay is identical to the assay with the radioactive substrate, and the substrate concentration remains at 200 μM (+)-ABA.

19. An additional modification to the protocol involves the switch from ethyl acetate extraction to the Oasis HLB column purification of metabolites. The sample assays are diluted to 1 mL after acidification and clarification. The Oasis HLB columns are secured in a holder with test tubes below to catch the waste. A vacuum manifold is attached and the vacuum set to 5 in. Hg. The columns are conditioned with 1 mL of methanol, equilibrated with 1 mL of water, and then 1 mL of sample is added. The column is washed with 1 mL of 5% methanol in water (v/v), and the tubes with waste are removed and replaced with clean tubes to catch the sample. The vacuum is reattached and the sample eluted with 1 mL of methanol. Samples are evaporated to dryness with a Vacufuge.
20. Nonradioactive assay products purified by either ethyl acetate extraction or with Oasis HLB columns are redissolved in methanol and analyzed by HPLC detection. ABA and its metabolites are separated on a Supelcosil HISEP reverse-phase column (150 × 4.6 mm) by isocratic elution with a 75:25 (v/v) mixture of 1% acetic acid (in water) and acetonitrile and UV detection at 262 nm. ABA and PA are identified by retention times, and quantitative measurements of the amounts of these compounds are calculated from peak areas by comparison with known quantities of (−)-PA and (+)-ABA (see Note 19). A calibration curve representing different amounts of PA or ABA is developed. The initial products of the catabolism of ABA, 8′-hydroxyABA and phaseic acid, are summed and expressed as nmoles product (PA) per mg protein over the duration of the reaction (usually 3 h) (see Note 20).

3.4. Modifications and Optimization of ABA 8′-Hydroxylase In Vitro Assay

The in vitro assay conditions, although reasonably simple and straightforward in this assay, were extensively optimized for substrate and cofactor concentrations, buffer, pH and temperature, extraction conditions, etc., to ensure maximum and reproducible activity from the experimental material. All of the initial conditions and protocols used to develop this assay were focused, as a first step, on generating some/any measurable amount of detectable enzyme activity (detectable product/phaseic acid formation). The assay was then optimized in a more systematic way to develop it as a useful and reproducibly quantitative assay (see Note 21).

1. The supernatant of the initial centrifugation contained the majority of the enzyme activity (as expected), and this crude fraction could have been used directly to monitor enzyme activity if less sensitivity had been required. This fraction was quite dilute for the enzyme activity and still contained many other soluble proteins and cell factors/metabolites from the initial extract, and thus the enzyme may not have retained its activity as long if this fraction had been used.

2. The most important factor in developing a high-activity (+)-ABA 8′-hydroxylase in vitro assay is the identification of a tissue expressing large amounts of in vivo activity. When this in vitro ABA 8′-hydroxylase assay was first developed (4), very few cytochrome P450s from plants had been functionally characterized; currently, there are approximately 50–60 plant cytochrome P450 enzymes with known activities. It should now be possible to develop in vitro assays for some of these enzymes to monitor substrate specificities and quantitative expression from tissues with high activities or tissues transformed to overexpress the enzyme, coupled with very sensitive product detection methods (20, 27).
3. The appearance of enzyme activity induced by (+)-ABA in corn suspension cells is transient (4, 28), and this should be considered in developing any such assays involving induction protocols. A time course of enzyme activity after induction should be considered and well-documented.
4. The choice of buffering agent and pH for the extraction buffer is critical in preserving activity. Much greater activity was always found with phosphate buffers rather than HEPES-based extraction buffers (Fig. 2). The efficiencies of these extraction buffers were compared through combinations of phosphate and HEPES at varying ratios (with or without EDTA and/or EGTA) (Krochko, unpublished data). The results suggest that HEPES is not inhibitory as an extraction buffer, but rather that phosphate exerts a concentration-dependent positive effect on activity that could be supplemented, but not entirely replaced by EDTA. Additionally, the pH and buffer choice for the assay buffer are important as well, and this should be determined empirically and confirmed once some initial enzyme activity has been established (Fig. 2). For the ABA 8′-hydroxylase enzyme from corn, both the K-phosphate and HEPES buffers are equally appropriate for the assay/resuspension buffer. The optimum temperature of the reaction is 32–38°C.
5. Neither EDTA nor an osmotic agent, i.e., sucrose, is required within the enzyme assay to maintain activity.
6. Losses of enzyme activity when the purified microsomal enzyme preparation is kept on ice over the course of a day are relatively minor (~20% over 6 h). In fact, induced enzyme activity is lost faster in the intact incubating (+)-ABA-induced suspension cultures over the course of a day than in the enzyme extracts (see Fig. 3a). Nonetheless, delays or interruptions in the extraction and purification of the microsomal enzyme preparation once the protocol is initiated could result in very substantial losses of enzyme activity. Any such delays should be rigorously avoided.

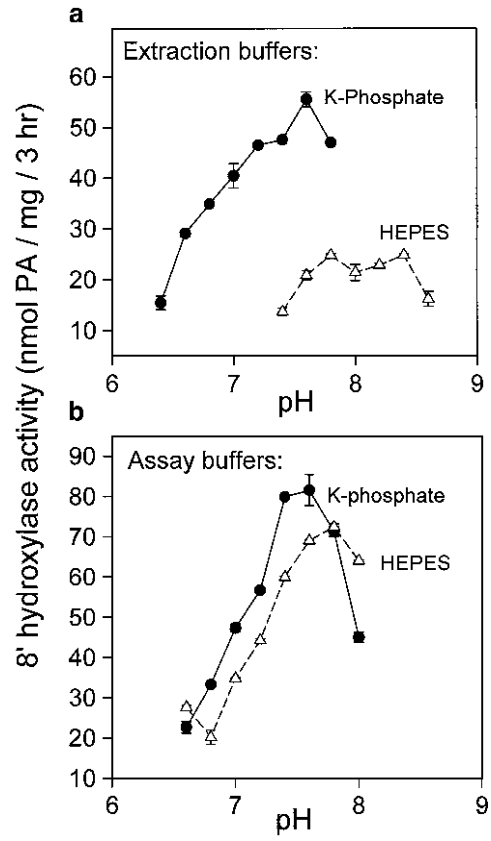

Fig. 2. Effect of buffers and pH on in vitro assayable ABA 8′-hydroxylase activity. (**a**) Comparison of the efficacy of 100 mM HEPES-NaOH and 100 mM potassium phosphate as the initial extraction buffers over a range of pH values. (**b**) Comparison of 100 mM HEPES-NaOH and 100 mM potassium phosphate as assay buffers over a range of pH values. The initial extraction was performed with 100 mM potassium phosphate buffer, pH 7.6. After ultracentrifugation, the microsomal pellets and the cofactor were resuspended for the assay in the buffers indicated at each data point on the graph. (Reproduced from ref. 4).

7. Enzyme activities can be compared to mRNA accumulations/concentrations across the time course of induction by northern blots (Fig. 3b). A tight correlation can be used to deduce a causal relationship between the enzyme activity and the gene/probe used for hybridization on the northern blot.
8. The choice of cofactor and concentration also are important. NADPH at high concentrations (5 mM) is required to maximize ABA 8′-hydroxylase activity in the assay. NADH does not adequately substitute for NADPH in supporting the enzyme activity; however, NADH does support ABA 8′-hydroxylase activity at suboptimal NADPH concentrations (Fig. 4) (4). This synergism could be exploited to reduce the costs of each reaction by reducing the amounts of the very expensive

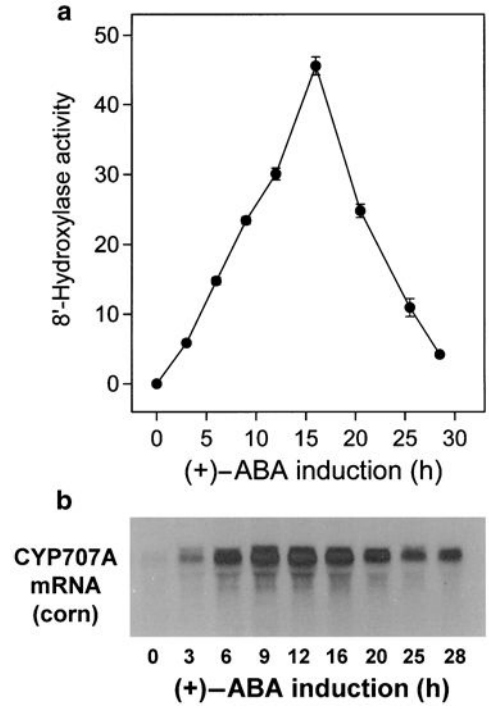

Fig. 3. Time course of the induction of ABA 8′-hydroxylase activity following the addition of (+)-ABA to BMS corn suspension cultures. Tissue was harvested at the times indicated after the addition of (+)-ABA to the culture medium. (**a**) Phaseic acid (PA) and 8′-hydroxyABA were assayed by HPLC and summed to represent the total ABA 8′-hydroxylase activity (expressed as total PA produced/mg protein/3 h). (**b**) Messenger RNA accumulation for ABA 8′-hydroxylase (CYP707A subfamily) was monitored through northern blots on total RNA using a probe to a corn CYP707A gene. (Adapted from ref. 4, and Krochko et al., unpublished).

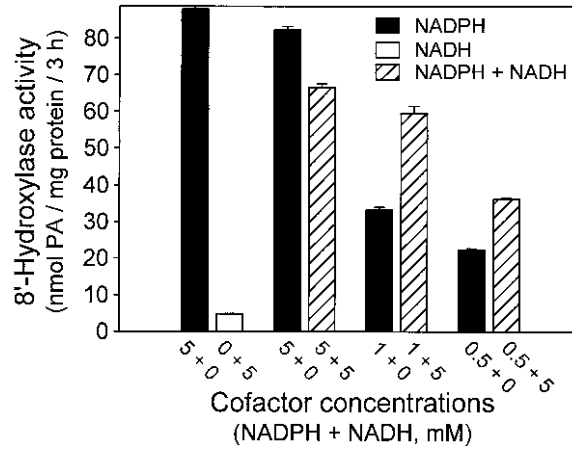

Fig. 4. The relative efficiencies of NADPH, NADH, and various combinations of these cofactors in supporting (+)-ABA 8′-hydroxylase activity. Concentrations: NADPH, 5, 1, 0.5 mM; NADH, 5 mM; and NADPH, 5, 1, 0.5 mM in combination with NADH at 5 mM. (Adapted from ref. 4).

NADPH required for each reaction. Alternatively, the NADPH could be replaced with an enzymatic NADPH-regenerating system which can be considerably cheaper, but which we did not try (available from BD Biosciences).

3.5. In Vitro Assay for ABA 8′-Hydroxylase from Tobacco Leaves

We were interested in developing a prototype in vitro assay for ABA 8′-hydroxylase using differentiated tissues from intact plants as a quantitative comparison to the corn suspension cells. We were able to demonstrate in vitro ABA 8′-hydroxylase activity in microsomes isolated from young seedlings of tobacco (*N. tabacum* cv. Xanthi) (Krochko, unpublished data). The standard in vitro assay with HPLC detection (described previously) is not sensitive enough in this case to detect products of the enzyme activity; however, the standard in vitro assay using [^{3}H]-(+)-ABA as the substrate (see Fig. 1a) and very long exposure to X-ray film (4.5 weeks at –80°C) of the TLC-separated ABA metabolites from the reaction provides clear evidence of phaseic acid production (see Fig. 5). It is estimated that the differences in enzyme activity between (+)-ABA-induced corn suspension cultures and the young tobacco leaves are in the order of a 1,000-fold difference (based on the intensity of the phaseic acid signals, the exposure time taken to develop a visible band for phaseic acid on the X-ray film, and the assumption that the extraction and assay conditions optimized for corn microsomes are also optimal for the tobacco microsomal preparations).

The enzyme activity we recorded using young tobacco leaf tissue is of a similar magnitude to the values reported previously for embryonic axes of chickpea (24). Using the detection methods described in this chapter, it is not possible to routinely sample this enzyme activity in microsomes from young tobacco leaves (or other such tissues). However, newer detection methods, i.e., LC MS/MS, that are more sensitive than using radiolabeled

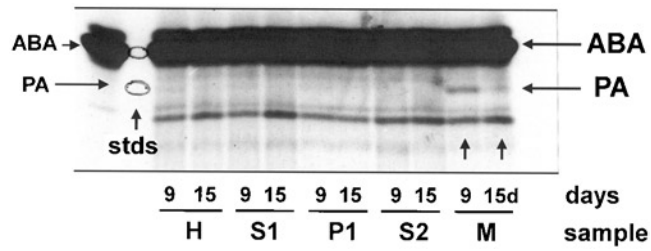

Fig. 5. Development of an in vitro assay for ABA 8′-hydroxylase from young tobacco leaves. Microsome preparations and the in vitro assay with [^{3}H]-(+)-ABA were conducted as described previously for corn tissues. Tobacco leaves were collected from 9- and 15-day-old seedlings. The TLC plate was exposed to the film for 4.5 weeks at –80°C for detection of phaseic acid by fluorography. H, tissue homogenate; S1, first supernatant; P1, first pellet; S2, second supernatant; M, microsomal pellet; PA, phaseic acid; ABA, abscisic acid. (Krochko et al., unpublished).

compounds now make it feasible to use in vitro assays more frequently to directly measure cytochrome P450 enzyme activities. This also permits more accurate comparisons with gene expression estimates based on northern blots, microarray, EST frequencies, and real-time RT-PCR. In fact, in vitro assays for direct measurements of cytochrome P450 activities in plant tissues have been used more frequently in recent years (20).

4. Notes

1. All solutions and media described in this chapter are made with distilled deionized water (other high-quality water can be substituted).
2. Chemically synthesized preparations of ABA are often available as racemic mixtures of the (+) and (−)-ABA stereoisomers. It is important to recognize that the natural form of ABA is S(+)-2-cis,4-trans-ABA, and other formulations, such as R(−)-ABA or (±)-ABA have weaker or diluted responses, and thus pose difficulties in correctly assigning the enzyme activity to the natural form of ABA.
3. The response of a plant tissue or enzyme extract to any solvent should be checked in order to confirm that there is no effect from the solvent alone. For some purposes, ABA can be dissolved in ethanol, methanol, or dimethyl sulfoxide (DMSO).
4. Other compounds are not required in the extraction buffer to maintain enzyme activity (e.g., catalase, protease inhibitors, chelating agents). For some plant tissues, it might be advisable to add protease inhibitors to this extraction solution.
5. When this protocol was published (4), we used 15-mL Corex tubes at 15,000 rpm (20,000 × *g*) for 10 min in an SS-34 rotor (with adaptors) for the first centrifugation. Current information on the 15-mL Corex tubes (now made by Kimble) specifies that the maximum RCF for these is 13,100 × *g*; and therefore, the speed indicated in the Methods section in this chapter has been changed to 12,000 rpm (13,100 × *g*) for 15 min. There are a number of plastic tubes with enhanced strength now available for high-speed preparative centrifugation, and further information on these can be obtained from various manufacturers.
6. The sequential dilutions of BSA described in making the protein standards for the protein assay are designed to maximize accuracy and reproducibility of these concentrations.

7. ABA is radiolabeled at six positions (3′, 5′, 5′, 7′, 7′, 7′) at high-specific activities. It is prepared using methods involving the exchange of hydrogen atoms on the ABA molecule with radioactive hydrogen atoms from radiolabeled water (29). This exchange reaction is encouraged under basic conditions, but requires appropriately licensed facilities and chemistry expertise. The product of the enzymatic reaction in the assay, phaseic acid, has the same labeling pattern, although it can be reduced through exchange reactions during purification and work-up of the sample. Stored samples of radiolabeled ABA are examined regularly by TLC to determine purity, and quantities of radiolabeled (+)-ABA are repurified by TLC if required (the purified [^{3}H]-(+)-ABA is eluted from scrapings of the TLC plate with ethanol).
8. The programmable Thermomixer (Eppendorf) is invaluable for these assays. This apparatus maintains the desired temperature (hotter or cooler than ambient) while gently shaking the assay tubes.
9. Large amounts of culture could be grown and induced for enzyme expression at one time. Large harvests allow for scale-up and reproducible comparisons of several treatments and/or chemicals at a time with the advantage that base activity for the enzyme is constant across all samples in that tissue lot.
10. Care is taken to prevent any warming of the tissue during this process. Ample liquid nitrogen is always present.
11. The chilled buffer frequently freezes slightly when added to the ground tissue in the mortar. This slightly frozen slurry is stirred gently with the pestle until it is thawed and then immediately strained through Miracloth.
12. It is very important to proceed rapidly to the isolation of the microsomes. All solutions, including the extraction buffer and enzyme solution, and all tubes are prechilled and kept chilled at all times.
13. The enzyme activity of the microsomal preparation is reasonably stable when stored on ice over the course of the day.
14. Other protein measurement assays can be used. This is dependent upon the additives (detergents, reducing agents) required in the extraction and resuspension buffers and their compatibilities with various protein assays (see Bio-Rad).
15. The standard assay period is 3 h, but this can sometimes be shortened to accommodate more samples. In either case, the appropriate reaction duration is determined empirically to cover the linear stage of the enzymatic reaction. Enzyme activity in our standard assays is based on the conversion or appearance of phaseic acid (from ABA) over a 3-h period (see Figs. 1–4).

16. Acidification of the assay mixture improves the extraction of ABA and its metabolites into ethyl acetate during the purification prior to TLC or HPLC.
17. The first product of the reaction, 8′-hydroxyABA, is not stable under these conditions and quickly cyclizes to phaseic acid. Any 8′-hydroxyABA detected in the assays (TLC or HPLC detection) is added to the phaseic acid to estimate/quantify the reaction rate through total product produced.
18. The chromatography runs are much improved using a wick (Whatman filter paper) along the back of the tank and allowing the sealed tank to equilibrate before running the TLC plate. These modifications prevent "smiling" of the bands.
19. Two assay extraction and two detection methods are provided for the benefit of the reader. The Oasis cartridges improve time efficiencies, allowing one to increase the number of samples handled and the size of the individual experiments. Likewise, HPLC detection is more efficient (though not as sensitive as the radioactive method) and permits quantitative comparisons between treatments.
20. We did not have a calibration curve for 8′-hydroxyABA because under these conditions this compound is transient, but we made the assumption that the response factor for 8′-hydroxyABA is the same as that for phaseic acid (the stable derivative).
21. It should be noted that in vitro assays from tissue-derived microsomal extracts cannot easily confirm or identify the specific gene/protein product (cytochrome P450 enzyme) responsible for the activity; rather, these assays only show that the enzyme activity is expressed in that tissue. The enzyme activity in question can be attributed to any one of the many P450 enzymes accumulating in that tissue. Confirmation of a specific enzyme activity for an individual cytochrome P450 gene may require heterologous expression in yeast, bacteria, or insect cultures and/or manipulated over- and underexpression in a host plant system.

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Chapter 9

Functional Analysis of Abscisic Acid 8′-Hydroxylase

Akira Endo, Mitsuhiro Kimura, Naoto Kawakami, and Eiji Nambara

Abstract

Abscisic acid (ABA) plays an important role in the control of seed dormancy and germination. Identification of hormone metabolism genes from a particular plant species of interest is an essential step in hormone research. The function of these gene products is validated by biochemical analysis using heterologous expression systems, such as *E. coli* and yeast. ABA 8′-hydroxylase is a subfamily of P450 monooxygenases and is encoded by *CYP707A* genes. CYP707A catalyzes the committed step in the major ABA catabolic pathway. In this chapter, we describe the methods for RNA extraction from seeds, cloning the CYP707A cDNAs, protein expression in yeast, and biochemical analysis of their gene products.

Key words: RNA extraction, cDNA cloning, Heterologous expression, P450, Seed, ABA 8′-hydroxylase

1. Introduction

The control of seed physiology by plant hormones is mediated through regulation of hormone metabolism and responsiveness. Abscisic acid (ABA) regulates seed dormancy and germination (1). Current advances in identifying ABA metabolism genes from the model plant *Arabidopsis* enable investigations into the diverse functions of this hormone in the seeds of various other plant species. Also, recent progress in genome- and EST-sequencing projects allows the design of primers for cloning of the orthologous genes from other plant species. Here, we describe the protocols for cloning and functional analysis of *CYP707A* genes for the study of seed physiology. These procedures are composed of four major steps (1) total RNA extraction from seed; (2) cloning of CYP707A cDNAs; (3) heterologous expression of plant CYP707A cDNAs in yeast; and (4) biochemical analysis of CYP707A enzymes. Below, we

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discuss the general aspects of these four major steps, and this is followed by the associated detailed methodologies.

1.1. Total RNA Extraction from Seeds

Extraction and purification of RNA from seeds rich in phenolics and polysaccharides or in lipids can be problematic. Polysaccharides contain similar physicochemical properties to those of nucleic acid polymers, and are often co-purified with RNA. It is difficult to remove polysaccharides from RNA once they are co-precipitated, so polysaccharides need to be separated from RNA in or before the first precipitation step. In general, chaotropic reagents, such as guanidine isothiocyanate and guanidine hydrochloride, which allow solubilization of polysaccharides in the extraction buffer, are not suitable for RNA extraction from seeds. A classical SDS-phenol method is usually adequate for RNA extraction from seeds, although this method also sometimes gives colored RNA, perhaps as a result of the RNA binding covalently to oxidized phenolics. This colored RNA is not suitable for reverse transcription and in vitro translation reactions. Many protocols have been developed to reduce such impurities by modifying the extraction buffer with a high concentration of salt to precipitate polysaccharides, with polyvinylpyrrolidone (PVP) and/or polyvinylpolypyrrolidone (PVPP) to adsorb phenolic compounds in combination with reducing agents, such as dithiothreitol (DTT), and with high ionic strength and high pH. Also, some of the extraction buffers contain cetyltrimethylammonium bromide (CTAB), which efficiently removes polysaccharides and inhibits polyphenol oxidase (e.g., (2)). An extraction buffer containing proteinase K without phenol extraction steps is reported to be effective for seeds (3). Protocols associated with commercial RNA extraction kits can be effective for RNA extraction from seeds, but sometimes they require considerable modification to obtain high-quality RNA (4). In Subheading 3, we describe a modified SDS-phenol method, which gives high-quality RNA for cDNA cloning, quantitative RT-PCR, and microarray analysis, especially from polysaccharide-rich seeds such as whole grains or the endosperm tissues of monocotyledonous species.

1.2. Cloning of CYP707A cDNAs

Total RNA is subjected to reverse transcription; the quality of RNA is essential for the success of this step. Subsequent PCR amplification needs a primer set to amplify the unidentified orthologues. Recent genome and EST data available at public databases allow for the design of primer sequences for cloning the orthologous genes. Table 1 shows examples of available *CYP707A* sequences from various plant species. In Subheading 3 below, we describe the procedures for cloning of the *CYP707A* genes for which sequences are available from public databases. Designing degenerated primers on the conserved regions and subsequent RACE-PCR is required when sufficient sequence data is not available (5). Sequencing the

Table 1
Examples of *CYP707A* genes identified from higher plants

Plant	Gene symbol	Number of identified genes	Accession number
Arabidopsis	AtCYP707A	4	At4g19230, At2g29090, At5g45340, At3g19270
Bean	PvCYP707A	3	DQ352541, DQ352542, DQ352543
Lettuce	LsABA8ox	4	AB235917, AB235918, AB235919, AB235920
Potato	StCYP707A	3	DQ206630*, DQ206631*, DQ206632*
Rice	OsABA8ox	3	Os02g0703600, Os08g0472800, Os09g0457100*
Barley	HvABA8′OH	2	DQ145932, DQ145933

Asterisks indicate that gene function is annotated by sequence homology, and enzyme function has not been shown

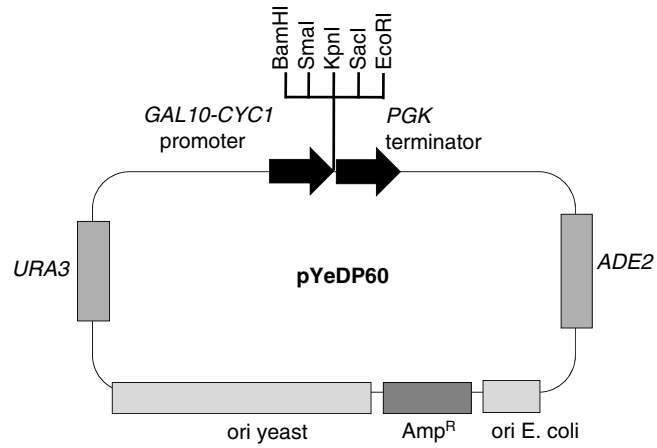

Fig. 1. The structure of pYeDP60 vector. This vector contains two selection markers, *URA3* and *ADE2*. WAT11 and WAT21 strains contain the *URA3* gene as a selection marker for integration of an *Arabidopsis* P450 reductase gene into the chromosome. Thus, *ADE2* is the functional selection marker in this expression system. The vector information is modified from Pompon et al. (6).

obtained CYP707A cDNAs is necessary to confirm that no undesirable mutations are generated during the cloning steps.

1.3. Heterologous Expression of Plant CYP707A cDNAs in Yeast

The resultant cDNA is subcloned into the plasmid pYeDP60 (6). pYeDP60 is a high-copy expression vector in yeast and contains an inducible *GAL10-CYC1* hybrid promoter for expressing a heterologous protein (Fig. 1). The cDNA cloned in pYeDP60 is transformed into a yeast strain, either WAT11 or WAT21 (6). WAT11 and WAT21 contain an *Arabidopsis* P450 reductase and ATR1 and ATR2, respectively (see Note 1). The *Arabidopsis* P450 reductase gene is integrated in the yeast chromosome, and the P450 reductase can be co-induced with a P450 by the *GAL10-CYC1* promoter.

The transformants are cultured, galactose-induced, and the microsome fraction is prepared as described below.

1.4. Biochemical Analysis of CYP707A Enzymes

The microsome fraction is used for the enzyme reaction. The reaction mixture is extracted with ethyl acetate and is analyzed by HPLC. Also, the product needs to be confirmed by mass spectrometry coupled with either GC or LC.

2. Materials

2.1. Total RNA Extraction from Seeds

2.1.1. Solutions

1. RNA extraction buffer: 200 mM Tris–HCl (pH 8.2), 100 mM LiCl, 50 mM EDTA, 1.5% (w/v) SDS. Store at room temperature. Add PVP (2%), PVPP (0.5%, insoluble), 1/50 volumes of 0.5 M DTT (10 mM), and mix just before use. The 0.5 M DTT stock solution can be stored at –20°C.
2. Phenol solution: 90% (w/w) phenol. Melt solid phenol with a heated water bath (40–60°C), then add 1/10 w/w water, and mix well. Store at room temperature.
3. Chloroform.
4. Lithium chloride solution 1: 10 M LiCl. Autoclave and store at 4°C.
5. Lithium chloride solution 2: 2 M LiCl. Autoclave and store at 4°C.
6. Ethanol solution: 70% (v/v) ethanol. Store at 4°C.
7. Sodium acetate buffer: 3 M sodium acetate (pH 5.2). Store at 4°C.
8. Ethanol solution: 95–99% (v/v) ethanol. Store at –20°C.

2.1.2. Equipment

1. Blender: Nissei AS-4 homogenizer.
2. Electronic coffee mill (optional). A mill with a separate mill chamber is ideal (e.g., Iwatani IFM-200D).

2.2. Cloning of CYP707A cDNAs

2.2.1. Kits, PCR Reagents, Restriction Enzymes, and Vectors

1. QuantiTect Reverse Transcription Kit (Qiagen).
2. PCR Reagents: Phusion High-Fidelity DNA polymerase and Phusion HF buffer (NEB), forward and reverse primers, and dNTPs.
3. Restriction enzymes: *Kpn*I, *Eco*RI, *Bam*HI (e.g., from NEB).
4. pGEM^{R}-T Easy vector system I (Promega).
5. pYeDP60 (see Note 2).

2.2.2. Solutions

1. PEG solution: 13% (w/v) PEG8000, 1.6 M NaCl.
2. Miniprep solution I: 50 mM glucose, 25 mM Tris–HCl (pH 8.0), 10 mM EDTA (pH 8.0), 100 μg/mL RNase A. Store at 4°C.

3. Miniprep solution II: 0.2 N NaOH, 1% (w/v) SDS.
4. Miniprep solution III: 3 M potassium acetate (pH 5.3).

2.2.3. Equipment

1. DNA Engine PTC-200 (MJ Research).

2.3. Heterologous Expression of Plant CYP707A cDNAs in Yeast

2.3.1. Strains and Transformation Kit

1. WAT11/WAT21 strain (see Note 2).
2. Frozen-EZ Yeast transformation II™ (Zymo Research)

2.3.2. Media

1. YEPD broth plus Ade: 1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose (see Note 3), 1% adenine in 0.1 N NaOH (4 mL for 1 L).
2. SGI plates: 1% (w/v) agar, 1% (w/v) glucose, 0.335% (w/v) yeast nitrogen base without amino acids, 0.05% (w/v) Bacto casaamino acids, 20 mg/L L-Tryptophan.
3. SLI broth: 2% (w/v) galactose (see Note 4), 0.67% (w/v) yeast nitrogen base without amino acids, 0.1% (w/v) Bacto casaamino acids, 20 mg/L L-Tryptophan.

2.3.3. Solutions

1. Potassium phosphate buffer (KPB): 0.1 M KPB (pH 7.6) (see Note 5).

   Add 8.2 g KH_2PO_4 in 600 mL distilled water and add 15.7 g K_2HPO_4 in 600 mL distilled water. KH_2PO_4 solution is added gradually to K_2HPO_4 until pH is 7.6.

2.3.4. Equipment

1. French Laboratory Press (Thermo Scientific).
2. Ultracentrifuge (Hitachi CP100WX).

2.4. Biochemical Analysis of CYP707A Enzymes

1. ABA standard: 30 μM (+)-*S*-ABA

   Add 1 ng/μL ethanol solution of (+)-*S*-ABA (see Notes 6 and 7), store at −20°C with aluminum foil.

2.4.1. Solutions

2. Hormone metabolite (phaseic acid, PA) standard: 30 μM (−)-phaseic acid

   Add 1 ng/μL ethanol solution of (−)-phaseic acid (see Notes 6 and 7) and store at −20°C with aluminum foil.
3. Nicotinamide Adenine Dinucleotide Phosphate (reduced tetrasodium salt) Solution:

   50 mM NADPH

   Dissolve NADPH in distilled miliQ water and cover with aluminum foil, store at −20°C.
4. HCL: 1 N HCl.
5. Ethyl acetate.

6. Solvent A: 10% (v/v) methanol (HPLC grade), 0.1% (v/v) acetic acid in distilled water (HPLC grade).
7. Solvent B: 60% (v/v) methanol (HPLC grade) and 0.1% (v/v) acetic acid in distilled water (HPLC grade).

2.4.2. Equipment

1. HPLC equipped with a photodiode array detector. 2996-600-717 system (Waters).
2. HPLC column; COSMOSIL 5C18-MS-II (4.6 i. d. × 150 mm, nacalai tesque).

3. Methods

3.1. Total RNA Extraction from Seeds

1. Two grams of seeds (e.g., approx. 50 wheat grains, which have large dead endosperms) are used as the starting material (see Note 8). Samples are frozen by liquid N_2 upon harvest and stored at –80°C. The frozen samples are preground by a coffee mill or suitable blender with liquid N_2 to facilitate grinding and ensure the yield of RNA. Then, the samples are ground to a fine powder with liquid N_2 using a prechilled mortar and pestle (see Note 9).
2. The resultant powder is transferred to a blender cup containing 20 mL of RNA extraction buffer, 15 mL of 90% phenol, and 5 mL of chloroform and then homogenized for 2 min at room temperature (see Note 10).
3. The aqueous phase (top) is separated from the phenol:chloroform phase and from cell debris (bottom) by centrifugation at 10,000 × *g* for 10 min at room temperature. The aqueous phase is taken carefully with a Pasteur pipette (see Note 11) and transferred to a new centrifuge tube with a screw cap containing 10 mL of 90% phenol and 10 mL of chloroform. The tube is shaken horizontally for 10 min at room temperature using a reciprocal shaker (see Note 12).
4. After the centrifugation at 10,000 × *g* for 5 min at room temperature, the aqueous phase is transferred to a new tube (or beaker) containing 20 mL of chloroform. The samples are homogenized for 10 min at room temperature as in step 3, followed by centrifugation for 5 min at room temperature (see Note 13).
5. The aqueous phase is transferred to a new tube, and is added a 1/4 volume of 10 M LiCl. The tube is stood overnight at 4°C to precipitate high-molecular-weight RNA including mRNA.

6. RNA is collected by centrifugation at 10,000 × *g* for 15 min at 4°C. The supernatant is discarded by decantation. The pellet and tube surface are washed with 5 mL of 2 M LiCl and then washed with 70% ethanol. The tube is centrifuged at 10,000 × *g* for 2 min at 4°C, and the supernatant is discarded by decantation carefully. The precipitate is air-dried and dissolved with 1 mL of autoclaved water. The resultant RNA solution is transferred to a smaller tube (e.g., a 15-mL tube) (see Note 14).
7. For ethanol precipitation, 0.1 mL (1/10 volumes) of 3 M sodium acetate and 2 mL (2 volumes) of 95% ethanol are added, mixed, and stood at −20°C for 1 h. RNA is collected by centrifugation at 10,000 × *g* for 10 min at 4°C. The supernatant is discarded, and the pellet is washed with ice-cold 70% ethanol (see Note 15). The pellet is air-dried either by standing the tube at room temperature or by using centrifuge evaporator. The dried pellet is dissolved in 50–100 μL of autoclaved water on ice. One to two microliters of the RNA solution is used for quantification. The RNA solution is diluted 100-fold with water, and the absorbance at 230, 260, and 280 nm is measured (see Note 16). Degradation of RNA during the extraction step can be checked by denatured agarose gel electrophoresis (see Note 17).
8. The samples are stored at −20°C (short term) or −80°C (long term) until use.

3.2. Cloning of CYP707A cDNAs

1. Total RNA (1 μg) is mixed with 2 μL of gDNA Wipeout Buffer (QuantiTect Reverse Transcription Kit) and made up to 14 μL with RNase-free water on ice. The mixture is incubated for 2 min at 42°C and placed immediately on ice to denature mRNAs. One microliter of Quantiscript Reverse Transcriptase, 4 μL of Quantiscript RT Buffer, and 1 μL of RT Primer mix (QuantiTect Reverse Transcription Kit) are added and mixed on ice. The reaction mixture is incubated for 15 min at 42°C. Reverse transcription reaction is stopped by heating at 95°C for 3 min.
2. CYP707A cDNAs are amplified by PCR with a set of *CYP707A*-specific primers (see Notes 18 and 19).
3. The resultant PCR product is PEG-precipitated by adding 100 μL of PEG solution and 80 μL of sterilized water into 20 μL of PCR products. After incubation at 4°C for 1 h, PCR products are collected by centrifugation at 10,000 × *g* for 20 min. The pellet is washed with 100 μL of 70% ethanol. The supernatant is discarded and the pellet is air-dried, resuspended in 20 μL TE buffer.
4. Two hundred nanograms of PCR products are mixed with 1 μL of linear pGEM-T Easy vector, 5 μL of 2× rapid ligation

buffer, 1 μL of T4 DNA ligase (3 Weiss units), and distilled water to a final volume of 10 μL. The reaction mixture is incubated at 25°C for 1 h. Two microliters of the ligation mixture is used for transformation into *E. coli*. *E. coli* competent cells are mixed with the ligation mixture, placed on ice for 30 min, heat shocked at 42°C for 1 min, and placed on ice for 10 min. Five hundred microliters of LB medium is added to the cells and incubated at 37°C for 1 h with agitation. The cells are plated out on an LB agar plate containing 50 mg/L ampicillin and incubated overnight at 37°C.

5. Approximately 24–48 colonies are used for colony PCR with M13 primers or gene-specific primers.
6. *E. coli* colonies are picked up, inoculated in 1.5 mL of LB medium containing ampicillin, and grown overnight with agitation at 37°C.
7. For miniprep, cells are harvested by spinning down for 30 s by centrifugation at 10,000 × *g*, and the supernatant is removed. One hundred microliters of solution I is added and vortexed. Subsequently, 200 μL of solution II is added and mixed by inverting the tube. The tube is placed on ice for 5 min. One hundred and fifty microliters of solution III is added, mixed by inverting the tube, and placed on ice for 10 min. Cell debris is removed by centrifugation at 10,000 × *g* for 10 min. The supernatant is collected and transferred to a new tube. The plasmid is collected by isopropanol precipitation by adding an equal volume of isopropanol. The tube is stood at room temperature for 10 min. After centrifugation at 10,000 × *g* for 10 min, the supernatant is discarded and the pellet is washed with 100 μL of 70% ethanol. The pellet is air-dried and resuspended in 20 μL TE buffer.
8. The sequences of resultant CYP707A cDNA clones are confirmed by sequencing. Five different restriction sites (*Bam*HI, *Sma*I, *Kpn*I, *Sac*I, and *Eco*RI) can be used for directional cloning into pYeDP60 (see Note 20).
9. Steps 4–7 are repeated using pYeDP60 (see Note 21). The subcloned cDNA is also confirmed by sequencing (see Note 22).

3.3. Heterologous Expression of Plant CYP707A cDNAs in Yeast

1. The yeast strain WAT11 is grown at 30°C in 1 mL of YEPD broth plus Ade until the mid-log phase (OD600 = ~0.8).
2. Cells are harvested by centrifugation at 500 × *g* for 4 min, and the supernatant is discarded. The cells are washed using 1 mL of EZ1 solution (Frozen-EZ Yeast transformation II™, Zymo Research). Cells are harvested by centrifugation at 500 × *g* for 4 min, and the supernatant is discarded. The cells are resuspended in 100 μL of EZ2 solution. Fifty microliters of the suspended cells in EZ2 solution is mixed with 2 μL of plasmid

DNA (~500 ng) and 500 μL of EZ3 solution. The cells are incubated at 30°C for 45 min, mixed vigorously, and plated (50–100 μL) on SGI plates.

3. After 2 days of incubation at 30°C, colonies are independently picked up and inoculated in 5 mL of SGI broth, followed by incubation at 30°C for 2 days.
4. Five milliliters of seed culture is inoculated in 250 mL of SGI broth in a 1-L flask and incubated at 30°C for 2 days with shaking (130 rpm).
5. Cells are harvested by centrifugation at 6,700 × *g* for 5 min at 20°C and washed with 100 mL of distilled water, resuspended in 250 mL of SLI broth in a 1-L flask, and incubated at 30°C for 12 h with shaking (130 rpm).
6. After the galactose induction, cells are harvested by centrifugation at 6,700 × *g* for 5 min at 20°C and the supernatant is discarded. The cells are resuspended in 25 mL of KPB and homogenized using a French Laboratory Press (1,000–1,500 psi).
7. Lysates are centrifuged at 10,000 × *g* for 15 min at 4°C to remove the debris. The supernatant is further centrifuged at 100,000 × *g* for 1 h at 4°C. The pellet containing microsomes is suspended in 0.5 mL KPB on ice with glass homogenizer. The resultant microsomal fraction is stored at −80°C until use.

3.4. Biochemical Analysis of CYP707A Enzymes

1. One hundred microliters of microsomal fraction is mixed with (+)-*S*-ABA (final concentration 30 μM) and NADPH (final concentration 0.5 mM) on ice. The reaction mixture is incubated at 22–30°C with agitation for an appropriate period (up to 12 h). The reaction is stopped by adding 10 μL of 1 N HCl.
2. The reaction mixture is mixed with 250 μL of ethyl acetate and vortexed vigorously. The reaction product is recovered from the ethyl acetate phase after centrifugation at 2,500 × *g* for 3 min at 4°C. The ethyl acetate phase is transferred to a new glass vial.
3. Step 2 is repeated twice.
4. The obtained ethyl acetate phases are evaporated using a SpeedVac. The dried pellet is dissolved by 100 μL of solvent A and kept on ice with an aluminum foil cover or stored in a −20°C freezer until HPLC analysis.
5. One-half of the extracted reaction product is subjected to reverse-phase HPLC (Fig. 2). Before and after sample analysis, the retention times of authentic ABA and phaseic acid are confirmed. Retention times for authentic ABA and PA are 14 and 8.5 min, respectively (see Note 23). The HPLC settings are:

Flow rate: 1.0 mL/min
UV detection: 254 nm
Solvent B

0–1 min	50%
1–10 min	50–100% linear gradient
10–14 min	100%
14–15 min	100–50%
15–18 min	50%

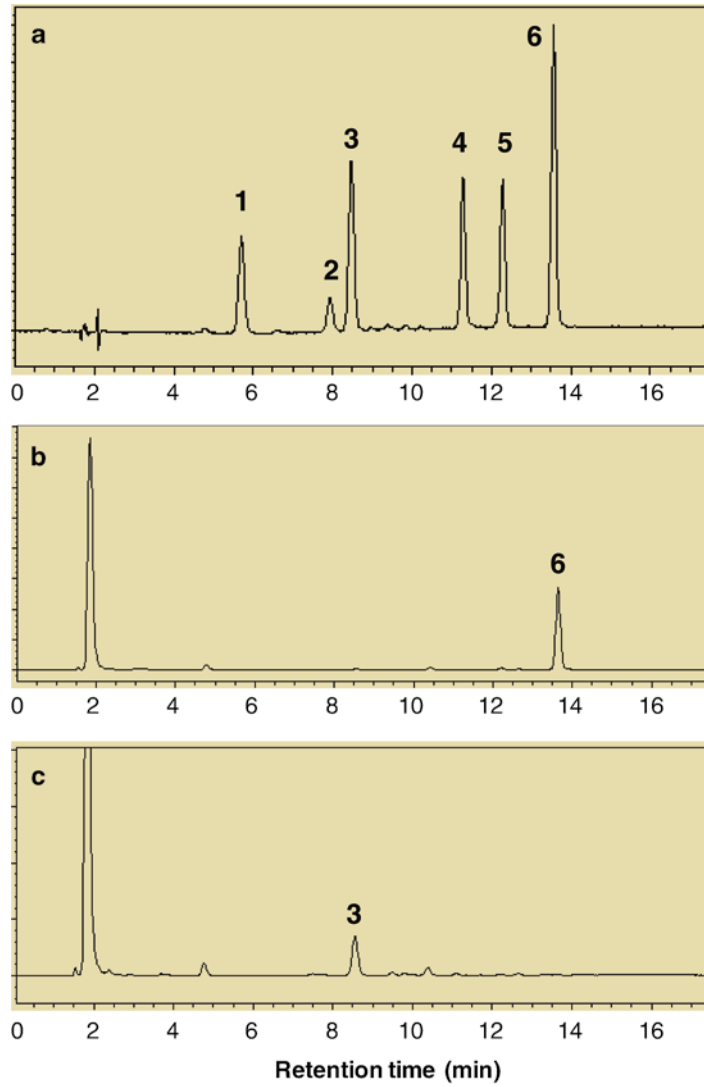

Fig. 2. HPLC data for an ABA 8′-hydroxylase of lettuce. (**a**) HPLC chart of authentic samples. (**b**) Reaction mixture with vector control. (**c**) Reaction mixture with LsABA8ox2, an ABA 8′-hydroxylase from lettuce (5). Peak 1, dihydrophaseic acid; peak 2, ABA glucose ester; peak 3, phaseic acid; peak 4, 7′-hydroxy ABA; peak 5, neophaseic acid; peak 6, ABA. *X*-axis and *Y*-axis indicate retention time (min) and absorbance (254 nm), respectively.

6. A portion (1/10 to 1/50) of the extracted reaction product is subjected to either LC-MS or GC-MS analysis (4, 7, 8).

4. Notes

1. Most of the tested CYP707A cDNAs show similar results when expressed in either WAT11 or WAT21.
2. Yeast strain WAT11/WAT21 and pYeDP60 can be obtained from Dr. Denis Pompon (CNRS, Centre de Genetique Moleculaire).
3. The 50% (w/v) glucose is filter-sterilized and added after autoclaving.
4. The 50% (w/v) galactose is filter-sterilized and added after autoclaving.
5. KPB is stored at 4°C.
6. The enantiomer of ABA, (−)-*R*-ABA, is not a substrate of known CYP707As. Therefore, (+)-*S*-ABA is a more effective substrate than racemic ABA. Long periods of light exposure convert ABA and PA to inactive *trans* isomers. Therefore, both ABA and PA (as dry compounds and solutions) need to be stored in darkness.
7. Authentic ABA catabolites, including PA, were obtained either from Dr. Susanne Abrams (National Research Council of Canada, Plant Biotechnology Institute) (9) or from Dr. Nobuhiro Hirai (Kyoto University) (10).
8. Take approximately 50 mg samples for extraction when using isolated embryos.
9. Wear leather gloves to prevent frostbite.
10. Wear plastic gloves to avoid phenol and chloroform contact with skin.
11. Do not take the white interphase.
12. Stirring in a beaker with a magnetic stirrer is also effective.
13. From this step, autoclaved or RNase-free materials should be used.
14. When you get a turbid solution, centrifuge at 10,000 × *g* for 5 min to remove undissolved impurities.
15. Ethanol remaining in the final RNA solution may inhibit the reverse transcription. The residual ethanol also interferes with electrophoresis by floating the samples when applied to the gel even after the addition of the loading buffer.
16. An RNA solution with high template activity usually has an OD_{260}/OD_{280} ratio of 1.7–2.0 and an OD_{260}/OD_{230} ratio above 2.4.

17. Ethidium bromide staining visualizes the two main cytosolic rRNA bands. The upper band (25S rRNA) may give a twofold more intense signal than the lower band (17S rRNA) when there is no degradation.
18. A standard PCR condition is as follows: denature at 95°C for 30 s and then conduct amplification with 30 cycles of 95°C for 10 s, 55°C for 30 s, and 72°C for 30 s, followed by a final extension for 3 min at 72°C. The PCR is carried out using 1 μL of 1/100 diluted cDNA solution.
19. Specific primers for *Arabidopsis CYP707A* genes are:

CYP707A1-F	5′-ATGGATATCTCCGCCTTGTTTCTCA-3′
CYP707A1-R	5′-CTAGTCTCCTCTTTCCAAATATAGA-3′
CYP707A2-F	5′-ATGCAAATCTCATCTTCATCGTCTT-3′
CYP707A2-R	5′-TTAAATCGGGGTTACTCTTATTGGT-3′
CYP707A3-F	5′-ATGGATTTCTCCGGTTTGTTTCTCA-3′
CYP707A3-R	5′-CTATGGTTTTCGTTCCAAGGCAATA-3′
CYP707A4-F	5′-ATGGCTGAAATTTGGTTCTTGGTTG-3′
CYP707A4-R	5′-CTAAAGAGAATGTCGACGAAATGTA-3′

20. The sequences of your cDNAs are to be confirmed whether they contain the restriction sites for *Bam*HI, *Sma*I, *Kpn*I, *Sac*I, and *Eco*RI for cloning into pYeDP60. The multicloning site of the pGEM-T also contains two *Eco*RI sites and one *Sac*I site.
21. The multicloning site of the pYeDP60 contains the restriction sites for *Bam*HI, *Sma*I, *Kpn*I, *Sac*I, and *Eco*RI as unique sites (Fig. 1). These enzymes can be utilized for directional cloning of the cDNA insert. Re-amplification of the cDNA by PCR using primers with the restriction site facilitates the directional cloning into pYeDP60.
22. Primers for confirming the insert cDNA in pYeDP60 are:

pYeDP60 seq-N	5′-GCATAAATTACTATACTTCTATAGAC ACGC-3′
pYeDP60 seq-C	5′-CCTTCCAATAATTCCAAAGAAGCA CCACCA CC-3′

23. It is essential to have control experiments, such as enzyme reactions with microsomes from yeast harboring an empty vector or without NADPH.

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Part IV

Gene, RNA, and Protein Analyses

Chapter 10

Isolation of Genetic Material from *Arabidopsis* Seeds

Urszula Piskurewicz and Luis Lopez-Molina

Abstract

Here, we describe a series of methods suitable for the reproducible and abundant isolation of total RNA, genomic DNA, and total protein from dry or imbibed *Arabidopsis* seeds. The resulting material is suitable for most standard molecular biology procedures.

Key words: Seed germination, RNA isolation, DNA isolation, Protein isolation, Protein transfer to membrane, cDNA synthesis, RT-PCR, Microarray analysis, Sodium bisulfite sequencing, Radioactive in vivo labeling, Immunopurification

1. Introduction

Embryonic material found in seeds is usually not optimally suited for the numerous DNA and RNA isolation kits available for purchase. The reasons are most likely due to the nature of seed material: seeds typically contain high concentrations of storage reserves (e.g., oils and carbohydrates) as well as high amounts of organic materials relative to water content (embryos have small vacuoles). These properties render purification of DNA, RNA, or protein more difficult if only because they require more extensive extraction steps; thus, the overall process is less efficient. Higher efficiency can be achieved in part by higher extraction volumes per amount of initial material, a configuration not optimal for commercial kit development, where speed and convenience are de rigueur. It is, therefore, not surprising that most of the kits tested in our laboratory often, if not always, yield poor results because the final yields are low or because the extracted material is not sufficiently clean or both.

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We have compiled here a number of basic techniques used on a routine basis in our laboratory, where seed material is the primary object of investigation. By "seed material" we mean either mature dry *Arabidopsis* seeds or *Arabidopsis* seeds that have been imbibed for a germination assay and are at stages prior to the young seedling stage (i.e., 48–72 h after seed imbibition under normal conditions). Nevertheless, we also routinely use the same techniques with young seedlings, giving comparable results, if not better, to those obtained with commercial kits.

All the protocols described here are derived from published work by colleagues in other laboratories. Our contribution is, therefore, mostly to compile and describe those approaches that have given in our hands consistent results over the years. We also have included some slight modifications from the original published protocols.

2. Materials

2.1. Total RNA Isolation from Arabidopsis Seed Material

1. Extraction buffer: 8 M LiCl, 2% β-mercaptoethanol.
2. Solubilization buffer: 0.5% SDS, 100 mM NaCl, 25 mM EDTA, 10 mM Tris–HCl, pH 7.6, 2% β-mercaptoethanol.
3. 10× ANE solution: 100 mM NaAc, 1 mM NaCl, 10 mM EDTA, adjust pH to 6.0 with HCl.
4. Chlorophane stock solution preparation:
   (a) Melt 1 kg of phenol (crystalline) at 65°C in the presence of 0.05% (w/v) 8-hydroxyquinoline (see Notes 1 and 2).
   (b) Saturate melted phenol two times with 1 M Tris–HCl (pH 8.0):
      (i) Add 500 mL of 1 M Tris–HCl (pH 8.0), stir for 1 h at room temperature.
      (ii) Stop stirring and allow phases to separate (adding 500 mL of H_2O helps separation).
      (iii) Aspirate off upper phase.
      (iv) Repeat the above procedures (i)–(iii) once.
   (c) Saturate melted phenol two times with 0.1 M Tris–HCl (pH 8.0): same as above, but stir for 30 min. At this point, pH should be above 7.0 (pH of phenol is below 5.0).
   (d) Add to the phenol phase one volume (500 mL) of chloroform.
   (e) Saturate the chloroform:phenol mixture with 1× ANE buffer:
      (i) Add 300 mL of 1× ANE buffer, stir for 30 min at room temperature.

(ii) Stop stirring and allow phases to separate.

(iii) Aspirate off upper phase.

(iv) Repeat the above procedures (i)–(iii) once.

(f) Aspirate off upper phase removing all, but approximately 2 cm of buffer.

(g) Store at 4°C. At this point, the pH of the chloroform:phenol mixture should be between 6.0 and 7.0.

2.2. Genomic DNA Isolation from Arabidopsis Seed Material

1. CTAB B solution: 1.4 M NaCl, 0.1 M Tris–HCl (pH 8.0), 2% (w/v) CTAB. Store at room temperature.
2. CTAB C solution: 50 mM Tris–HCl (pH 8.0), 10 mM EDTA (pH 8.0), 1% (w/v) CTAB. Store at room temperature.
3. CTAB D solution: 50 mM Tris–HCl (pH 8.0), 10 mM EDTA (pH 8.0), 1 M CsCl, 1% (w/v) CTAB. Store at room temperature.

2.3. Standard Protein Isolation for Immunoblot Analysis

1. 2× SDS gel loading buffer: 4% SDS, 200 mM dithiothreitol (DTT), 20% glycerol, 100 mM Tris–HCl (pH 6.8).

2.4. Acetone Precipitation of Proteins for Immunoblot Analysis

1. AP extraction buffer: 4% SDS, 2% β-mercaptoethanol, 20% glycerol, 100 mM Tris–HCl (pH 8.0).

2.5. Optimization of Protein Transfer to Membrane for Immunodetection

1. Glycine transfer buffer: 39 mM glycine, 48 mM Tris base, 0.037 % SDS w/v (electrophoresis grade), 20% methanol (v/v). Adjust pH to 8.3.
2. Carbonate transfer buffer: 4.4 mM Na_2CO_3, 12.5 mM $NaHCO_3$. Adjust pH to 9.9 with NaOH.
3. Diffusion transfer buffer: 50 mM NaCl, 2 mM EDTA, 0.1 mM DTT, 10 mM Tris–HCl (pH 7.0).

2.6. In Vivo Labeling of Proteins

1. Liquid Murashige and Skoog (MS) medium: MS basal salt mixture (Sigma) 3.4, 0.5 g/L MES (4-morpholineethanesulfonic acid), adjust pH with KOH to 5.7.

2.7. Immunopurification of Protein for Radiodetection or Mass Spectrometry Analysis

1. Lysis buffer: 0.5% (w/v) SDS, 0.05 M Tris–HCl (pH 8.0), 1 mM DTT freshly added.
2. RIPA correction buffer: 1.25% (w/w) Nonidet P-40 (NP-40), 1.25% (w/v) sodium deoxycholate, 0.0125 M sodium phosphate (pH 7.2), 2 mM EDTA, 0.2 mM sodium vanadate freshly added from 0.2 M stock solution, 50 mM sodium fluoride, 100 U/mL aprotinin.
3. RIPA lysis buffer: 1% (w/w) NP-40, 1% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 0.15 M NaCl, 0.01 M sodium

phosphate (pH 7.2), 2 mM EDTA, 0.2 mM sodium vanadate freshly added from 0.2 M stock solution, 50 mM sodium fluoride, 100 U/mL aprotinin (commercially available protease and phosphatase inhibitor cocktail may also be used).

3. Methods

3.1. Total RNA Isolation from Arabidopsis Seed Material (Adapted from Vicient and Delseny (1))

1. Freeze seed material (maximum 100 mg of dry seeds) with liquid N_2 and grind with mortar and pestle (a pinch of quartz helps disrupt the seed tissue).
2. Transfer material to 2-mL microcentrifuge tubes and add 2 mL of ice-cold extraction buffer.
3. Vortex mixture thoroughly and leave samples on ice overnight.
4. Centrifuge tubes for 4 s and collect supernatant, avoiding as much as possible insoluble debris.
5. Transfer supernatant to a clean 2-mL microcentrifuge tube and centrifuge at 18,000 × *g* for 30 min at 4°C.
6. Remove supernatant and vortex pellet in 75% ice-cold ethanol.
7. Centrifuge at 18,000 × *g* for 5 min at 4°C.
8. Remove supernatant and resuspend pellet in 500 µL solubilization buffer.
9. Extract RNA twice with an equal volume of chlorophane:
   (i) Add 500 µL of chlorophane.
   (ii) Vortex vigorously for at least 30 s.
   (iii) Centrifuge at 18,000 × *g* for 5 min at room temperature.
   (iv) Transfer supernatant to a fresh microcentrifuge tube.
   (v) Repeat the procedures (i)–(iv) once.
10. Add NaCl to a final concentration of 0.35 M and two volumes of 100% ethanol.
11. Let RNA precipitate for 20 min at –20°C.
12. Centrifuge at 18,000 × *g* for 15 min at 4°C.
13. Remove supernatant and wash pellet in 75% ethanol.
14. Centrifuge at 18,000 × *g* for 5 min at 4°C.
15. Remove supernatant and resuspend pellet in 50–70 µL of water.
16. Quantify RNA concentration by measuring absorbance at 260 nm (OD_{260}) using a spectrophotometer. RNA isolated from dry seeds (100 mg) is typically at a concentration of 100–300 ng/µL; when isolated from 100 mg of seeds collected 36–48 h after imbibition, it can be as much as 1 µg/µL

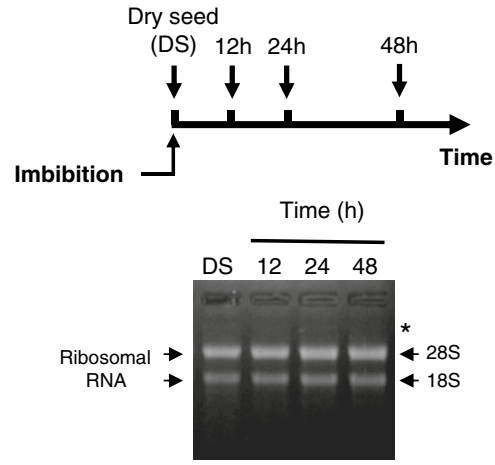

Fig. 1. Total RNA was extracted from wild-type (WT) (Columbia) *Arabidopsis thaliana* dry seeds and from imbibed seeds at the indicated times. RNA concentration was determined by spectrophotometric absorbance at 260 nm (OD_{260}). RNA (2 µg) was subjected to electrophoresis in an agarose/formaldehyde gel containing ethidium bromide. Note the equal loading per lane and the absence of fluorescence in the wells. Traces of genomic DNA are sometimes visible as a faint band in the upper parts of the gel (*).

(see Notes 3 and 4). An example of the results produced using this method is shown in Fig. 1.

3.2. Preparing Total RNA for Quantitative RT-PCR or Microarray Analysis

1. To 1 µg of total RNA prepared as described in Method 3.1, add 0.5–1 U of RQ1 DNase and 0.1 U of RNase inhibitor. Ensure that the final volume of the reaction is at least ten times higher than the combined volume of DNase and RNase inhibitor in the reaction (see Notes 5 and 6).
2. Incubate reaction at 37°C for 30 min.
3. Clean the reaction using commercially available spin columns according to the manufacturer's recommendations (e.g., Qiagen, RNeasy).
4. An even cleaner option prior to step 3 is to further extract the RNA once with one volume of chlorophane. Figure 2 shows typical fluorescence scans of purified total RNA as well as the resulting labeled complementary RNA (cRNA) prior to microarray hybridization.

3.3. Genomic DNA Isolation from Arabidopsis Seed Material (Adapted from Dellaporta et al. (2))

1. Freeze seed material (maximum 100 mg of dry seeds) with liquid N_2 and grind with a mortar and pestle.
2. Transfer material to 2-mL microcentrifuge tubes and add 750 µL of CTAB B solution.
3. Incubate for 10 min at 65°C with occasional vortexing.
4. Add 500 µL of a 1:1 mixture of CTAB B and CTAB C.
5. Incubate for 10 min at 65°C with occasional vortexing.

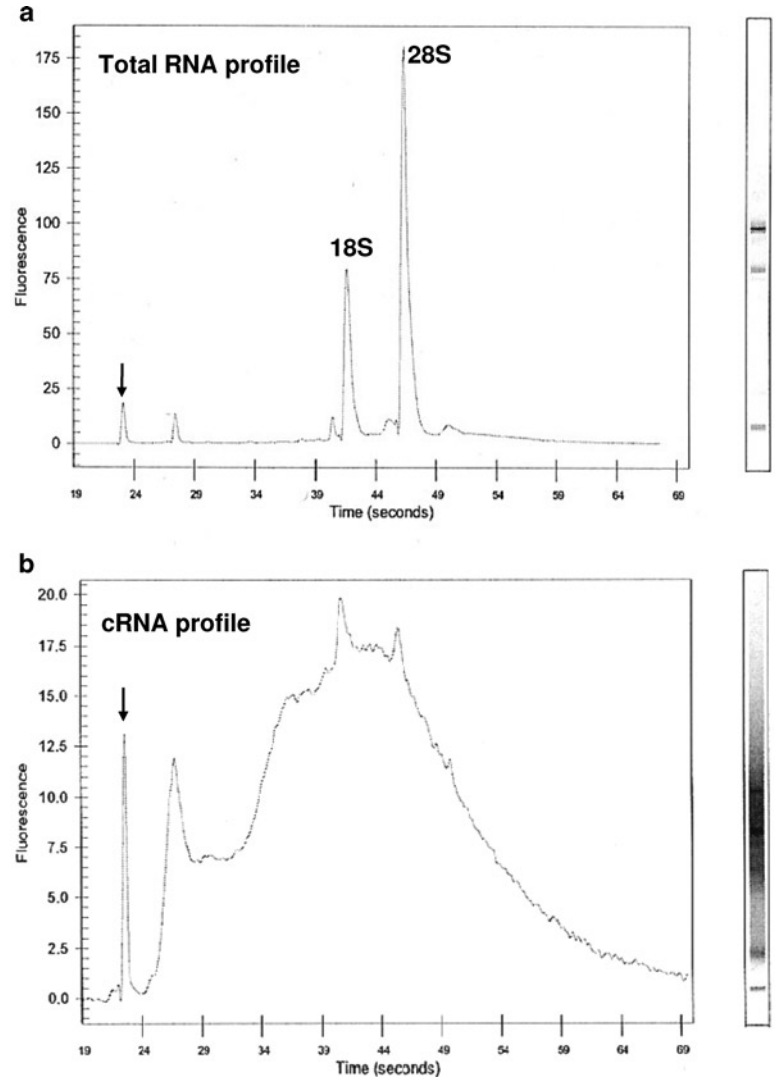

Fig. 2. (**a**) Agilent bioanalyzer scan of purified total RNA from WT (Ws) seeds 36 h after imbibition in the presence of 3 μM abscisic acid (thus preventing their germination). The fluorescence profile shows a characteristic signature of a high-quality total RNA sample. A 28S:18S rRNA ratio of 2.3 was calculated by integrating the areas of 18S and 28S rRNA peaks and then dividing the area of the 18S rRNA peak into the area of the 28S rRNA peak. The arrow indicates a peak corresponding to a marker RNA of 25 bases. (**b**) Agilent bioanalyzer scan of complementary RNA (cRNA). cRNA was linearly amplified from cDNA synthesized from 2 μg of total RNA shown in A using a QuickAmp Labeling Kit (Agilent) according to the manufacturer's instructions. The arrow indicates a peak corresponding to a marker RNA of 25 bases.

6. Centrifuge at 18,000 × *g* for 15 min at room temperature.
7. Transfer the supernatant to a clean tube and add 500 μL of chlorophane or chloroform.
8. Mix contents gently, but thoroughly, and centrifuge at 18,000 × *g* for 5 min at room temperature.

9. Repeat steps 7 and 8 once.
10. Transfer the upper phase to a fresh tube and add one volume of CTAB C. Mix contents gently, but thoroughly.
11. Incubate for 30 min at room temperature.
12. Remove supernatant and resuspend pellet in 500 µL of CTAB D supplemented with 1 µg of RNase A.
13. Incubate for 30 min at 37°C.
14. Add two volumes of 100% ethanol.
15. Let DNA precipitate for 20 min at –20°C.
16. Centrifuge at 18,000 × *g* for 5 min at room temperature.
17. Remove supernatant and wash pellet in 75% ethanol.
18. Centrifuge at 18,000 × *g* for 5 min at 4°C.
19. Remove supernatant and resuspend pellet in 50 µL TE (optional: pellet can be resuspended in 200 µL TE for an additional chlorophane extraction followed by an NaCl/ethanol precipitation).
20. Estimate DNA concentration by measuring absorbance at 260 nm (OD_{260}) using a spectrophotometer. At this point, DNA concentrations are in the range of 200 ng/µL to 1 µg/µL (see Note 7).

3.4. Standard Protein Isolation for Immunoblot Analysis (Laemmli (3))

1. Freeze seed material (maximum 50 mg of dry seeds) with liquid N2 and grind with pestle in an equal volume of 2 × SDS gel loading buffer (see Note 8).
2. Vortex mixture thoroughly and boil it for 10 min.
3. Centrifuge at 18,000 × *g* for 5 min at room temperature.
4. Quantify protein concentration using the Bradford dye assay or by measuring spectrophotometric absorbance at 260 nm (OD_{280}).
5. Add the bromophenol blue solution to a final concentration of 0.02% (v/v) (see Note 8).

3.5. Acetone Precipitation of Proteins for Immunoblot Analysis

Acetone precipitation of proteins can dramatically improve the signal-to-noise ratio in subsequent immunoblot analysis (see Fig. 4) and in some cases, it can make the difference between antigen detection versus absence of detection. This is illustrated in Fig. 5, where ABA-insensitive3 (ABI3), a seed-specific transcription factor, is detected only when proteins from seeds are prepared according to the acetone precipitation procedure.

1. Freeze seed material (maximum 100 mg of dry seeds) with liquid N_2 and grind with mortar and pestle.
2. Transfer material to 2-mL microcentrifuge tubes and add 0.4-mL AP extraction buffer.
3. Vortex mixture thoroughly and boil for 3 min.

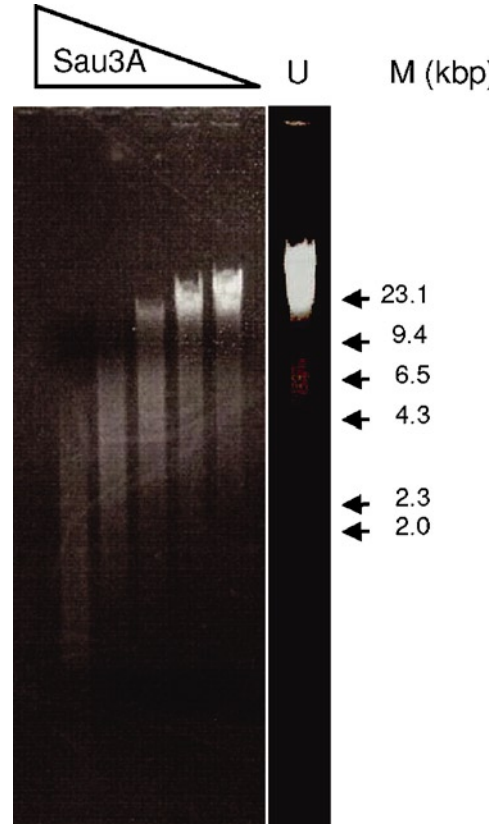

Fig. 3. Total DNA was extracted from WT (Ws) *A. thaliana* seeds 24 h after imbibition. DNA (1 μg) was digested with serial dilutions of the restriction enzyme Sau3A (GATC) for 15 min and then subjected to electrophoretic analysis in a 0.7% agarose gel containing ethidium bromide. U: undigested DNA (3 μg), M: Marker DNA.

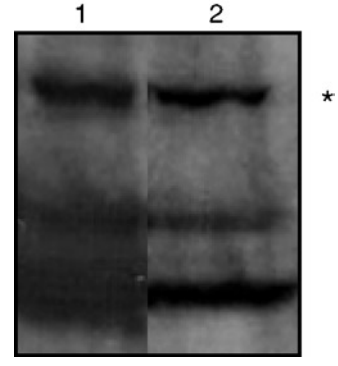

Fig. 4. Western blot analysis of GA-insensitive (GAI) and RGA (repressor of GA1-3) protein accumulation in *A. thaliana* (Col) paclobutrazol (PAC)-treated seeds harvested 48 h after imbibition. Lane 1: Total protein extract isolated using standard protein isolation protocol. Lane 2: Total protein extraction was followed by acetone precipitation of proteins. Ten microgram of total protein was loaded per lane; an aspecific band(*) serves as a loading control.

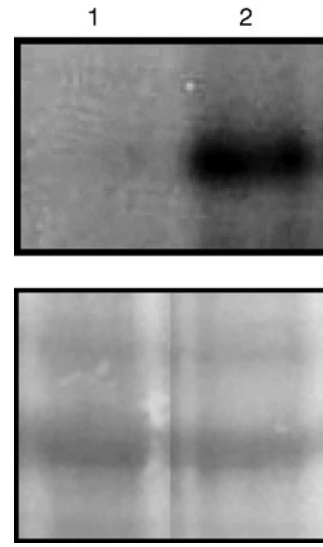

Fig. 5. Western blot analysis of ABA-insensitive3 (ABI3) protein levels in *A. thaliana* (Col) dry seeds. Lane 1: Total protein extract isolated using standard protein isolation protocol. Lane 2: Total protein extraction according to the acetone precipitation procedure. Ten microgram of total protein was loaded per lane.

4. Centrifuge at 18,000 × *g* for 10 min at room temperature and collect supernatant, avoiding as much as possible insoluble debris.
5. Transfer supernatant to a clean 2-mL microcentrifuge tube and precipitate proteins by adding four volumes of acetone.
6. Mix the components thoroughly and let proteins precipitate for 20 min at –20°C.
7. Centrifuge at 18,000 × *g* for 10 min at 4°C.
8. Remove supernatant and wash pellet with 80% acetone (see Note 9).
9. Remove supernatant and resuspend pellet in 100–300 µL of 2× SDS gel loading buffer (see Note 10).
10. Quantify protein concentration using the Bradford dye assay or by measuring spectrophotometric absorbance at 260 nm (OD_{280}).
11. Add the solution of bromophenol blue to a final concentration of 0.2% (v/v) (see Note 10).

3.6. SDS-Polyacrylamide Gel Assembly

1. Prepare an SDS-polyacrylamide gel according to standard procedures (4).
2. Centrifuge protein samples at 18,000 × *g* for 5 min at room temperature.

3. Load protein samples on the SDS-polyacrylamide gel avoiding as much as possible insoluble debris in pellet.
4. Run protein samples according to standard procedures (4).

3.7. Optimization of Protein Transfer to Membrane for Immunodetection

For most applications, protein transfer in a standard glycine buffer according to standard procedures (4) is sufficient for optimal detection of most antigen-antibody complexes. However, in some cases, a significant improvement can be achieved by using different protein transfer protocols. In place of the glycine buffer, a carbonate-containing buffer can be used; the procedure for transfer using this buffer is as described in (4). Figure 6 shows stronger recognition of ABI3 antigen using the carbonate buffer transfer approach versus the standard approach.

Below, we describe the diffusion transfer procedure, which is yet another alternative to the glycine buffer and carbonate buffer transfer approaches. Regardless of the protein transfer procedure, for antigen detection, the membranes should be treated according to standard procedures (4) or according to the particular requirements of the antibody used for optimal antigen detection.

1. Transfer by diffusion:
   (a) Transfer proteins to immunodetection membrane by submarine assembly in diffusion buffer (see Note 11) according to standard procedures (4), except that the polyacrylamide gel can be sandwiched between two nitrocellulose filters (see Note 12).

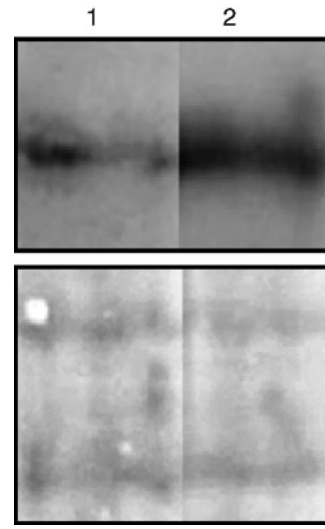

Fig. 6. Western blot analysis of ABA-insensitive3 (ABI3) protein levels in *A. thaliana* (Col) dry seeds. Lane 1: Total protein extract was transferred in glycine buffer. Lane 2: Total protein extract was transferred in carbonate buffer. In both samples, protein extracts were obtained using the acetone precipitation procedure. Ten microgram of total protein was loaded per lane.

(b) Place the assembled sandwich in the diffusion buffer in a 50°C bath.

(c) Let proteins transfer overnight by diffusion.

(d) For antigen detection, treat membrane according to standard procedures (4) or according to the particular requirements of the antibody used for optimal antigen detection (see Note 12).

3.8. In Vivo Labeling of Proteins

1. Prepare a 5-cm-diameter Petri dish containing nylon filter (mesh diameter approximately 50 μm) soaked in 0.6 mL of liquid MS media (see Note 13).
2. Plate 50–100 mg of dry seeds on nylon filter (see Note 14).
3. Prepare 0.6 mL of liquid MS media supplied with 20 μCi of (^{32}P)orthophosphoric acid.
4. Transfer nylon with imbibed seeds at the appropriate stage on the new Petri dish supplied with MS and (^{32}P)orthophosphoric acid.
5. Put Petri dish into vacuum bell and apply vacuum for 10 min at room temperature.
6. Incubate seeds for 2–3 h on MS media supplied with (^{32}P) orthophosphoric acid (see Note 15).
7. Harvest seeds into 10-mL tubes and wash them extensively with 8 mL of liquid MS media. Repeat the washing step five times to remove unincorporated radioactivity.
8. Freeze material with liquid N_2 and continue with procedure described below.

3.9. Immunopurification of Proteins for Radiodetection or Mass Spectrometry Analysis

1. Grind frozen seed material (maximum 100 mg of dry seeds) in liquid N_2 with mortar and pestle.
2. Transfer material to 2-ml microcentrifuge tubes and add 1-mL lysis buffer.
3. Homogenize the material in a 7-mL ground glass homogenizer.
4. Vortex mixture thoroughly and boil it for 5 min.
5. Transfer the mixture into a 10-mL tube and add four volumes of RIPA correction buffer.
6. Centrifuge tubes for 20 min at 4°C at 3,200 × *g* and collect supernatant, avoiding as much as possible insoluble debris.
7. Transfer supernatant to a fresh tube, add agarose/sepharose beads according to the manufacturer's recommendations, and continue with immunoprecipitation steps according to standard procedures, including initial washing steps with RIPA lysis buffer (5, 6).

4. Notes

1. One kilogram of crystallized phenol gives approximately 500 mL of melted phenol. Add 0.25 g of 8-hydroxyquinoline per 1 kg of phenol.
2. 8-hydroxyquinoline strongly absorbs light at 260 nm; therefore, it is important not to add more than the indicated amount.
3. This procedure normally allows quantitative determination of RNA concentration by measuring spectrophotometric absorbance at 260 nm (OD_{260}) irrespective of the physiological status (e.g., germination state) of the seed.
4. This procedure can be used for up to 100 mg of dry seeds as starting material (roughly a volume of about 100 μL). Beyond that amount, quality and yield decrease significantly.
5. The total RNA extraction procedure described in Subheading 3.1 often yields genomic DNA contamination. This simple purification procedure can be applied to obtain total RNA suitable for quantitative RT-PCR, cDNA synthesis, or as starting material for cyanine 5-labeled cRNA synthesis suitable for microarray hybridizations (see Fig. 2).
6. A typical reaction assemblage for preparing total RNA for quantitative RT-PCR or microarray analysis is as follows:

   20 μg total RNA

   13 μL RQ1 DNase (1 μ/μL)

   2 μL RNase inhibitor (1 μ/μL)

   H_2O to 150 μL
7. Genomic DNA isolated with this protocol is suitable for DNA blot analysis, sodium bisulfite sequencing (7), and for genomic library construction. Figure 3 shows a preparative series of restriction digests of genomic DNA isolated 24 h after dry seed imbibition using the frequent cutter Sau3A. Partially digested fragments were gel-isolated and used for packaging into phage heads for genomic library construction (8).
8. If protein quantification is not essential (as when harvesting equal sample amounts of seed material), protein extraction can be directly performed in the 2× SDS extraction buffer supplied with 0.02% bromophenol blue. Omit steps 4 and 5.
9. Step 8 can be repeated up to three times to improve the quality of the final protein sample.
10. If protein quantification is not essential (as when harvesting equal sample amounts of seed material), the 2× SDS extraction buffer in step 9 can already be supplemented with 0.02% bromophenol blue. Omit steps 10 and 11.

11. Two diffusion buffers can be prepared by buffering either with 10 mM Tris–HCl (pH 7.0) or with 10 mM glycine–NaOH (pH 11.0).
12. An advantage of this method is that proteins present in the same SDS-polyacrylamide gel can be transferred to two membranes giving two mirror images of the same protein samples. The disadvantage of the diffusion approach is that lower protein amounts are transferred per membrane. However, this method is particularly useful when the amount of protein extract is not limiting for the recognition of antibody. Indeed, transfer by diffusion can become a standard transfer method when detecting peptide-tagged antigens.
13. When a larger Petri dish is used, the volume of MS media has to be increased so that all seeds are in contact with media but are not fully submerged in liquid.
14. Depending on the experimental requirements, seeds can be sterilized and plated on MS agar plates before being transferred, at the appropriate time, into the media containing (^{32}P) orthophosphoric acid.
15. This step has been optimized for labeling and recognition of HA-ABI5 (5). This step has to be optimized for the particular target protein being studied.

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Chapter 11

Identification and Characterization of Quantitative Trait Loci that Control Seed Dormancy in *Arabidopsis*

Leónie Bentsink and Maarten Koornneef

Abstract

Seed dormancy is a trait that is under multigenic control and affected strongly by environmental factors. Thus, seed dormancy is a typical quantitative trait. Natural accessions of *Arabidopsis thaliana* exhibit a great deal of genetic variation for seed dormancy. This natural variation can be used to identify genes controlling this trait by means of quantitative trait loci (QTL) mapping. In this chapter, we describe how QTL mapping for seed dormancy in *Arabidopsis thaliana* can be performed and how QTL analyses can be used to eventually identify the causal gene. Methods and recourses available specifically for *Arabidopsis* are described or referred to.

Key words: Seed dormancy, *Arabidopsis*, Natural variation, Quantitative trait locus, Recombinant inbred lines, Near-isogenic line

1. Introduction

Seed dormancy is genetically a complex trait. It is under multigenic control and affected by environmental factors during seed development, seed storage, and seed germination. To identify genes controlling this trait, several strategies can be used. A classical approach is the study of induced mutants. For *Arabidopsis thaliana* (referred to as *Arabidopsis* hereafter), the standard laboratory accessions (Landsberg *erecta (Ler)*, Columbia, and Wassilewskija) have been treated with mutagens (such as the chemical ethyl methane sulphonate (EMS) and gamma irradiation) or subjected to insertions of T-DNA. In these genetic backgrounds, screens have been performed in order to identify nondormant mutants or mutants with reduced dormancy. These types of analyses revealed many mutants in which seed dormancy is affected (reviewed in (1, 2)).

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Another approach to identify genes controlling traits is by using natural variation. Genetic differences between natural accessions affect many traits. Usually, these traits are controlled by several genes that are strongly affected by the environment. These characteristics make them typical quantitative traits. The genetic analysis of quantitative traits corresponds to the identification, location, and genetic characterization of genomic regions, the so-called quantitative trait loci (QTL). This procedure is commonly referred to as QTL analysis or QTL mapping.

Arabidopsis is very suitable for studying natural variation, especially for seed dormancy where the common laboratory genotypes show limited seed dormancy in contrast to many other accessions. *Arabidopsis* is a wild species with a wide geographical distribution covering a diversity of environments with a large intraspecific natural genetic variation for many traits. Currently, the genomic resources available in *Arabidopsis* allow the analysis of this source of genetic variation up to the molecular identification of the underlying genes and polymorphisms (3).

The main prerequisite for QTL analysis is the availability of a mapping population. Typical QTL mapping populations are derived from F1 hybrids obtained by intercrossing two different *Arabidopsis* natural accessions that usually differ for the trait of interest. From this, a segregating population is established, which provides the foundation of genetic linkage analysis. Many kinds of mapping populations can be used for QTL analysis, including F2 and backcross (BC) populations. However, populations of recombinant inbred lines (RILs) have been the main choice in *Arabidopsis* due to the advantages derived from their homozygosity and their increased effective recombination. RILs are considered immortalized or permanent populations that can be propagated indefinitely via seeds and need to be genotyped for segregating markers only once. RIL populations can be used to map QTL very efficiently because the influence of the environment on the quantitative trait can be much reduced by assessing multiple individuals of the same genotype instead of just a single plant. Furthermore, once they have been obtained and genotyped, RIL populations can be used in an unlimited number of studies of different traits in different environments, allowing an efficient comparison of QTL positions obtained for different traits or in different conditions (comparative QTL mapping). The latter also allows for the detection of genotype–environment interactions. For *Arabidopsis*, a large number of RIL populations have been developed and genotyped and are publicly available (Table 1).

Another type of immortal mapping populations are introgression lines, in which a chromosome segment of one accession is introgressed in the background of another accession. Thus, far three complete sets of such lines have been published for *Arabidopsis* (4, 5), as well as a more limited collection with mainly whole

Table 1
Some Internet resources for analysis of natural variation, QTL, and confirmation of QTL in *Arabidopsis*

Content	Title	Internet address
Genotypic and phenotypic information on *Arabidopsis* collections of natural accession, RIL, and NIL populations	Natural variation INRA-Versailles Weigelworld	http://www.naturalvariation.org/ http://www.inra.fr/internet/Produits/vast/ http://www.weigelworld.org/research/projects/naturalvariation
Arabidopsis seed stock centers	ABRC NASC SASSC	http://www.arabidopsis.org/abrc/ http://www.nasc.nott.ac.uk/ http://www.brc.riken.jp/lab/epd/SASSC/
Arabidopsis markers and polymorphisms	TAIR Marker Search Cereon Polymorphisms MASC SNP Database MSQT – Multiple SNP Query Tool The genealogy of *Arabidopsis thaliana*: Nordborg lab dCAPS Finder 2.0 Marker Tracker POLYMORPH	http://www.arabidopsis.org http://www.arabidopsis.org/Cereon/index.jsp http://www.genomforschung.uni-bielefeld.de/GF-research/GABI-MASC.html http://www.msqt.weigelworld.org http://www.walnut.usc.edu/2010/pilot http://www.helix.wustl.edu/dcaps/dcaps.html http://www.bbc.botany.utoronto.ca/markertracker/index.spy http://www.polymorph.weigelworld.org/cgi-bin/retrieve_snp.cgi
Genetic mapping software	Alphabetic list of genetic analysis software Joinmap MAPMAKER, MAPMAKER/QTL Map Manager QTX MapQTL MultiQTL PlabQTL QTL Cartographer QTLexpress	http://www.nslij-genetics.org/soft http://www.kyazma.nl/index.php/mc.JoinMap/ http://www.broad.mit.edu/science/software/software http://www.mapmanager.org http://www.kyazma.nl/index.php/mc.MapQTL/ http://www.multiqtl.com http://www.uni-hohenheim.de/~ipspwww/soft.html http://www.statgen.ncsu.edu/qtlcart http://www.qtl.cap.ed.ac.uk

(continued)

Table 1
(continued)

Content	Title	Internet address
T-DNA insertion lines	ATIDB: The *Arabidopsis thaliana* Integrated Database	http://www.atidb.org/
	RARGE	http://www.rarge.gsc.riken.jp/dsmutant/index.pl
Tool for molecular cloning	Simplified *Arabidopsis* transformation protocol	http://www.entomology.wisc.edu/~afb/protocol.html
	Vapor-phase sterilization of *Arabidopsis* seeds	http://www.entomology.wisc.edu/~afb/vapster.html

chromosome substitutions (6). The advantages and disadvantages of both types of immortal mapping populations have been described by Keurentjes et al. (4).

QTL mapping requires the genotyping of experimental populations with markers every 5–10 centiMorgans to obtain the corresponding genetic maps. Marker techniques, such as polymerase chain reaction (PCR)-based markers, including microsatellites and codominant-cleaved amplified polymorphic sequences (CAPSs) or any of the high-throughput technologies currently available to detect single nucleotide polymorphisms (SNPs) or insertion/deletions (INDELs), can be used. Currently, a nearly unlimited number of SNP markers can be obtained from the resequencing of 876 fragments in 96 accessions (7) or from the nearly full genomes of many accessions ((8); http://www.polymorph.weigelworld.org/). These sequences can be surveyed to search for nucleotide polymorphisms using software programs specifically developed for this purpose, such as MSQT (9), and the new parental accession can then be tested for polymorphisms (Table 1). In addition, large collections of microsatellite markers (10–12), INDELs (13), and single-feature polymorphisms (SFPs; (14)) are available (http://www.arabidopsis.org), which can be genotyped with single-marker techniques or high-throughput methods, such as microarray hybridization (15).

Genotyped markers are arranged in a genetic linkage map based on their pairwise recombination frequencies. Such genetic data result in a genetic linkage map that provides the basis for the subsequent QTL analysis. For this, several statistical tools are used aiming to estimate the genetic locations and effects of QTL, their possible interactions with other QTL (epistasis) or with the environment, and their pleiotropic effects on other traits. The power of QTL detection and the accuracy of QTL location depend on the intrinsic characteristics that determine the genetic basis of the trait. These include the number of QTL, heritability of the trait, distribution of the effects of individual loci, interactions among QTL, and the linkage between QTL. In addition, the quality of QTL mapping also depends on several factors that can be partly manipulated in the experimental design, such as the type of mapping population, population size, number of observations per genotype, marker density of the linkage map, and statistical method used to map QTL. Due to the current interest in the analysis of *Arabidopsis* natural variation, several biological and genomic resources that are specifically devoted to this have been generated. These include collections of natural accessions, many RIL mapping populations, and several polymorphism databases involving various natural accessions (see Table 1). These resources make QTL analysis in *Arabidopsis* an efficient routine methodology that can be used to identify the major effect loci determining natural variation for any trait of interest.

To fully understand the natural genetic variation, after the QTL analysis of a trait, one needs to pursue the molecular characterization of the identified QTL. This includes the molecular isolation of the genes underlying individual QTL (referred to as quantitative trait genes, QTGs) and the identification of DNA polymorphisms altering the function of the gene and causing the phenotypic variation (the so-called functional polymorphisms or quantitative trait nucleotides; QTNs). The characterization of individual QTL requires their separation from the rest of the segregating loci. This can be done by constructing near-isogenic lines (NILs) that ideally differ only at a single locus (or several closely linked loci) affecting the trait of interest. These NILs can be derived by recurrent backcrossing of RILs. Alternatively, NILs can be obtained in a heterogeneous genetic background using heterogeneous inbred families (HIFs) derived from plants heterozygous for a particular region of interest in an otherwise homozygous background (16, 17). The HIF strategy is less time consuming in comparison to the traditional NIL construction. When NILs are backcrossed to their recurrent parent, the offspring of such crosses segregate only for the heterozygous region, in principle allowing the Mendelian analysis of a targeted QTL. This might include the QTL fine mapping and the study of interaction effects between QTL. In addition, NILs can be used for the further genetic, physiological, and molecular characterization of the QTL in a similar way as it is done with mutants. Nevertheless, the molecular isolation of QTL still requires a major effort (18) mainly due to the large amount of nucleotide diversity existing among natural accessions; two *Arabidopsis* accessions reveal on average six polymorphisms per kilobase of DNA (8, 19), which hampers the identification of causal QTGs and QTNs. Several genomic resources available in *Arabidopsis* are making QTG and QTN identification feasible (3). These resources include efficient fine or high-resolution mapping technologies, genome-wide gene expression tools, collections of insertional or EMS mutants for almost every *Arabidopsis* gene, complementation tests by plant transformation, and in the near future the possibility of full sequence comparison allowing association studies in small genomic regions (20) (Table 1).

1.1. QTL Analysis for Seed Dormancy

Many QTL for seed dormancy have been identified in crop and model plants (reviewed in (1)). For *Arabidopsis*, natural variation for seed dormancy has been studied in several RIL populations (21–25). QTL analysis for seed dormancy in the L*er*/Cape Verde Island (Cvi) RIL population (22) led to the identification of loci that had significant effects and that could be confirmed using NILs. A QTL mapping approach to identify loci affecting the after-ripening requirement measured as the number of *D*ays of

*S*eed *D*ry *S*torage required to reach *50%* germination (DSDS50) was used. Thus, seven QTL were identified and named *Delay of Germination* (*DOG*) *1–7*. To confirm and characterize these loci, NILs carrying Cvi introgression fragments in an L*er* genetic background were constructed. The analysis of these lines for germination confirmed four QTL (*DOG1*, *DOG2*, *DOG3*, and *DOG6*). The same type of analysis in the L*er*/Shahdara (Sha) RIL population revealed four QTL (23). Three of them were the already identified *DOG1*, *DOG3*, and *DOG6* (22); in addition, a QTL at chromosome 2 was identified. Recently, Laserna et al. (24) reported on natural genetic variation for the light responses of seeds of L*er*/Cvi and Bayreuth-0 (Bay-0)/Sha RIL populations and their interactions with after-ripening and incubation temperatures before light treatments. The authors identified 12 loci under red light (R), far-red light (FR), or darkness in both RIL populations. These include the previously identified *DOG1*, *DOG2*, *DOG3*, and *DOG7* (22) and five additional novel loci (*DOG8*, *DOG9*, *DOG13*, *DOG14*, and *DOG15*) mapped in the L*er*/Cvi RIL population. In addition, three loci (*DOG10*, *DOG11*, and *DOG12*) were identified in the Bay-0/Sha RIL population. Moreover, *DOG-LF1* and *DOG-LF2* were mapped in the low-fluence response (LFR) promotion of seed germination, and *DOG-VLF1* was mapped in the very-low-fluence response (VLFR) promotion of seed germination. In another study, Meng et al. (25) identified three major loci responsible for the variability in cold-tolerant and dark germination in the Bay-0/Sha RIL population. One of these QTL, *C*old-tolerant *D*ark *G*ermination (*CDG-1)*, was localized at the same genomic region as the delay of germination loci *DOG2* and *DOG3* (22) as well as a QTL that affects controlled seed deterioration and germination speed (23).

The first of these seed dormancy QTL, *DOG1* which had been identified in three of the analyzed populations, has been cloned. For this cloning, an NIL containing a Cvi introgression at the position of the QTL was used. *DOG1* is a member of a small gene family of unknown molecular function. It has not been previously associated with seed dormancy (26), indicating that natural variation can be valuable to identify novel seed dormancy genes.

In this chapter, we describe QTL analysis in *Arabidopsis* targeted to seed dormancy. We also describe the confirmation of the identified QTL by the construction of NILs and the eventual cloning of the QTG. Figure 1 shows QTL identified for after-ripening requirement in a total of six RIL populations, all made by crossing different accessions with L*er*, and the confirmation of some of these QTL using NILs (27). The formal theory behind QTL analysis is beyond the scope of this protocol chapter and can be found in textbooks (28, 29).

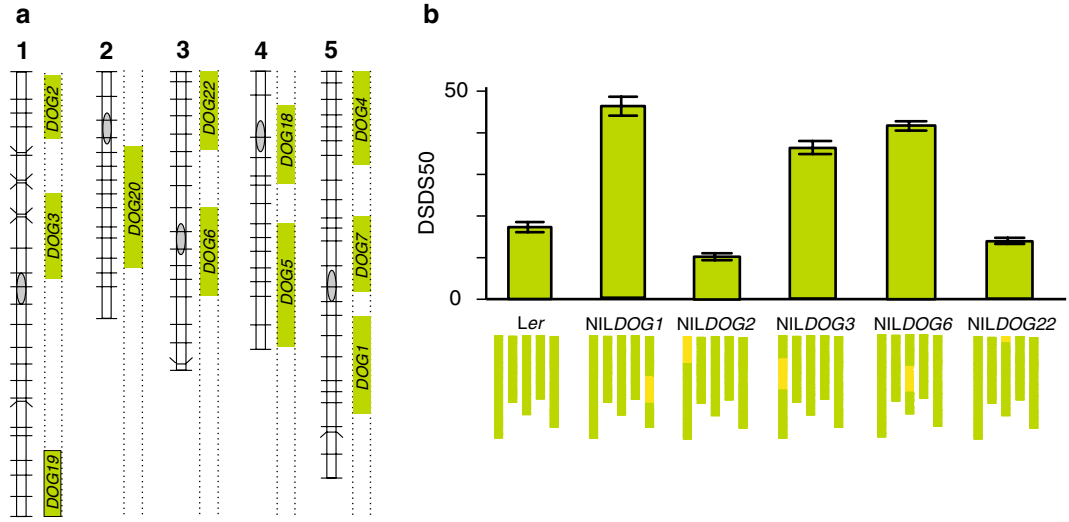

Fig. 1. Analyses of seed dormancy using natural variation. (**a**) Physical map showing the QTL positions for seed dormancy (*DOG1-11*; *grey bars*) identified in a total of six recombinant inbred line populations. (**b**) Schematic presentation of a subset of the NILs with their dormancy behavior in *D*ays of *S*eed *D*ry *S*torage required to reach 50% of germination (DSDS50) (27).

2. Materials

2.1. Seed Dormancy Analyses

1. Filter paper (Whatman 1, Whatman Kent, UK or Satorius 3HW, Satorius Stedim Biotech, Aubagne, France), Petri dishes, demineralized water, and stereo microscope.

2.2. QTL Analyses

1. Seeds of *Arabidopsis* accessions and mapping populations.
2. Equipment to cross plants (tweezers, stereo microscope, and labels).
3. Equipment to grow simultaneously, under the assay conditions, many plants necessary to perform the quantitative analysis of whole accession collections or mapping populations. Specific requirements depend on the particular test conditions.
4. DNA isolation protocol, preferably high throughput.
5. Equipment and materials to genotype molecular genetic markers. This depends on the technique used, but typically includes oligonucleotides and PCR ingredients, a thermocycler and an agarose gel system, for standard microsatellites and CAPS markers. It may further include equipment and reagents for high-throughput genotyping of SNPs or INDELs.
6. Software for general statistical analysis (e.g., SAS, SPSS, or STATISTICA packages), for linkage mapping analysis (e.g., MapMaker or JoinMap), and for QTL analysis (e.g., MapQTL or QTL Cartographer) (Table 1).

2.3. Confirmation of the QTL (Fine Mapping and Complementation Cloning)

1. Equipment to cross plants (tweezers, stereo microscope, and labels).
2. Equipment to grow simultaneously, under the assay conditions, many plants (see above).
3. Equipment to genotype molecular genetic markers (see above).
4. Cloning equipment (proofreading Taq polymerase, (binary) vector, antibiotics, *Agrobacterium tumefaciens*, sequencing facilities (30, 31)).
5. Transformation requirements (32), Simplified *Arabidopsis* Transformation Protocol (Clough and Bent, entomology.wisc.edu/~afb/protocol.html).
6. Equipment to sterilize seeds according to the chosen protocol (i.e., Vapor-Phase Sterilization of *Arabidopsis* Seed, Clough and Bent, entomology.wisc.edu/~afb/vapster.html).

3. Methods

3.1. Analyses of Seed Dormancy

Levels of seed dormancy in general are tested by evaluating germination percentages over time or in different germination conditions (e.g., those that vary in terms of temperature, light conditions, and the nature of the growth medium). The level of seed dormancy depends very much on environmental conditions during seed development, during storage, and during germination experiments. Therefore, it is essential that the physiological "history" of different genotypes that are compared are the same. Germination assays can be performed as follows:

1. Grow under appropriate assay conditions and in a random design, 4–20 individuals per genotype, and harvest the seeds (see Notes 1 and 2).
2. Conduct germination experiments to assign the level of seed dormancy. Germination tests in water under white light are performed by incubating seeds during 1 week as follows: between 50 and 100 seeds of a genotype are evenly sown on a filter paper soaked with 0.7 ml demineralized water in a 6-cm Petri dish. Petri dishes are placed in moisture chambers consisting of plastic trays containing a filter paper saturated with tap water and closed with transparent lids. Moisture chambers are stored for 1 week in a climate chamber at 22–25°C with a 16-h photoperiod (see Notes 3–5).
3. Germination percentages can be determined after the study period (e.g., 7 days), but also during the study period (e.g., every

day or every 2 days) to determine the germination rate that might be correlated with the level of seed dormancy. Germination tests are performed at different time points after seed harvest until 100% of the seeds germinate (see Note 6).

4. Germination curves can be made by plotting germination percentage as a function of time. Two parameters can be deduced from these curves: (a) DSDS50 and (b) *R*ate of *D*ormancy *R*elease (RDR) (see Note 7).

3.2. QTL Analyses

3.2.1. Selection of Parental Accessions

When initiating the analysis of natural variation for a trait of interest, it is useful to first explore the variation present for the trait(s) and its heritability (degree to which a characteristic is determined by genetics). Performing a preliminary general quantitative genetic study with available accessions determines whether a QTL analysis could be successful and it improves the choice of the parents and the design of the resulting QTL mapping experiment. To accomplish the above:

1. Grow and phenotype a collection of natural accessions as described in Subheading 3.1 (see Note 8).
2. Perform a quantitative analysis of the data and estimate the overall genetic variance, environmental variance, and heritability of the trait. If several traits have been measured in the same assay, correlations between the traits can be estimated to determine whether they have a common genetic basis.
3. The study can be completed by repeating the analysis under different environmental conditions, which might identify more suitable environments and/or accessions that respond differently to the environment (see Notes 9 and 10).
4. Choose the two parental accessions (see Notes 11 and 12) and design the experimental setup (quantitative assay, environmental conditions, population type, population size, and the number of replications per genotype) for the QTL analysis.

3.2.2. Construction of QTL Mapping Populations

When the accessions chosen for QTL analysis are not included in any of the existing mapping populations, a new mapping population must be developed. Here, we explain briefly the development of an RIL population (33).

1. Grow together the two parental accessions so that they flower at the same time, make a (reciprocal) cross between them by hand pollination, and harvest the F1 generation seeds.
2. Grow the F1 plants, check using morphological or molecular markers if they yield the expected result of the intended cross, and self them to obtain the F2 population seeds.
3. To obtain an RIL population, grow the F2 population and harvest the F3 self-progeny seed of each F2 individual plant.

The size of an RIL population is usually between 100 and a few hundred lines, derived from the same number of F2 plants.

4. Grow one (or several) randomly chosen F3 seed derived from each F2 individual and harvest the F4 self-progeny seed.
5. Repeat the previous single-seed descent procedure up to the F8/F9 generation. Thereafter, plants are practically homozygous and become lines that might be bulk harvested. Analyses can also be performed at earlier generations. At these stages, there is much more heterozygosity which allows one to obtain HIFs (see Subheading 3.5.2).

3.3. Development of Genome-Wide Linkage Maps

After a new mapping population has been constructed, it needs to be characterized genetically with molecular markers to obtain its molecular linkage map. The genotypic information of markers distributed throughout the genome and ordered according to the linear DNA structure of the five *Arabidopsis* chromosomes provides the frame for QTL mapping. To develop a genome-wide genetic map:

1. Grow the RIL population and the corresponding parental natural accessions, and isolate the DNA of the individual plants. To propagate the mapping population, obtain and harvest the self-progeny seeds of all the individuals.
2. Determine the marker density desired for the linkage map, search for potential markers, and test the parental accessions for the DNA polymorphisms that are used as molecular markers. Tools in order to find and develop markers can be found in Table 1.
3. Analyze the DNA of all the individuals of the mapping population for the molecular markers.
4. Score the genotype of all the individuals of the mapping population for each analyzed marker taking into account: the type of marker, parental origin of the segregating alleles (the linkage phase), and doubtful or missing data. Name the marker genotypes according to the instructions of the linkage mapping software to be used (see Table 1).
5. Enter the individual genotype scores of the molecular markers in a spreadsheet and build the appropriate locus genotype data file for the corresponding mapping software.
6. Use the mapping software to perform the linkage analyses and construct the genetic map.
7. The linkage map might be completed by including new markers within the genetic intervals flanked by adjacent markers distanced more than 10–15 cM.

3.4. Mapping QTL

A mapping population for which its genetic map has been developed can be used to identify and locate QTL accounting for the variation present in the population for the trait(s) of interest.

1. Grow in a random design and under the appropriate test conditions two to four blocks containing three to six individuals of each of the RILs and the parental accessions. Harvest the seeds and analyze them for dormancy as described in Subheading 3.1 (see Notes 1–7 and 13).
2. Perform a "classical" quantitative genetics analysis of the data to estimate the heritability of the trait(s) in the mapping population and the overall dominance and the maternal genetic effects of the trait(s) (see Note 14). Correlations between traits measured in the same mapping population might be estimated, which may include data publicly available from other experiments using the same mapping population (see Table 1).
3. Combine the quantitative data file with the molecular marker and linkage map files according to the instructions of the QTL mapping software to be used.
4. Process the data of each trait separately to identify the QTL affecting each trait of interest by using single-marker mapping, (composite) interval mapping, or any other QTL mapping method implemented in the software used. Determine the map position (QTL support intervals), the additive allele effect, and the percentage of explained variance of the individual QTL detected; estimate the total percentage of the phenotypic variance explained by all QTL.
5. Analyze the epistatic interactions among QTL and perform a comparative QTL analysis to identify QTL with putative pleiotropic effects (see Note 15).
6. The study can be completed by repeating the QTL mapping analysis under different environmental conditions to identify QTL that interact with environmental factors.

3.5. Confirmation of QTL

3.5.1. Confirmation Using NILs

1. Select an RIL that contains the required allele at the position of the QTL region. Cross this RIL to the accession (recurrent parent) in which background the allele needs to be studied.
2. Grow both lines together so that they flower at the same time, make a (reciprocal) cross between them by hand pollination, and harvest the F1 generation seeds.
3. Grow the F1 plants, check with morphological or molecular markers if they are the expected result of the intended cross, and self them to obtain the F2 population seeds.
4. Grow the F2 population (see Note 16) and the recurrent parent. Isolate DNA of all F2 plants and check the genotype. Harvest the F3 self-progeny seeds of the F2 plant that has the required genotype and those of the recurrent parent (see Note 17).
5. Check the F3 progeny for their seed dormancy phenotype by performing a germination experiment (described in

Subheading 3.1) and compare the results of this to that of its recurrent parent (see Note 18).

3.5.2. Confirmation Using HIFs

QTL can also be confirmed using an "HIF" strategy (16, 17). With this approach, NILs are selected from an inbred line that is not entirely homozygous. Progeny of this line segregates for those loci not yet fixed and represents HIF of nearly isogenic individuals.

1. At the F5–F7 stage, RILs can be selected that are homozygous everywhere on their genome, except for a small region around a marker at the position of the QTL of interest. Plant and genotype approximately 20 plants of these RILs, and then select two plants fixed for each of the two parental alleles (seeds from these plants are then used for further phenotyping; see Note 19).

3.6. Cloning of the QTL

3.6.1. Fine Mapping of the QTL

Fine mapping is performed in order to precisely define the position of the QTL. For the fine mapping, Mendelian segregation is used.

1. Select the NIL that contains an introgression for the QTL that you want to develop a fine mapping population for and the recurrent parent (see Note 20). When using the HIF strategy, the selected heterozygous RIL can directly be used for fine mapping (in that case, continue at step 4).
2. Grow both lines together so that they flower at the same time, make a (reciprocal) cross between them by hand pollination, and harvest the F1 generation seeds.
3. Grow the F1 plants, check with morphological or molecular markers if they are the expected result of the intended cross, and self them to obtain the F2 population seeds.
4. Grow the F2 population (see Note 21) and both parents (NIL and recurrent parent). Isolate DNA of all F2 plants and both parents. Genotype the F2 plants with a genetic marker at both borders of the introgression. Select plants that contain a cross-over between these markers. Harvest the F3 self-progeny seeds of the selected F2 plants and those of both parents.
5. Check the F3 progeny for their seed dormancy phenotype by performing a germination experiment (described in Subheading 3.1) and compare the results of this to that of its recurrent parent. Classify the F2 phenotype as deriving from each of the parents (see Notes 18, 22, and 23).
6. Compare the F2 genotypes to their respective phenotypes to see if the results are consistent (see Note 18). When this is the case, the QTL region can be reduced by adding a marker in between two first markers; by comparing the phenotype to the genotype, it can be decided at which site of the marker the

QTL is located. This process can be repeated until there is no or just one recombinant left between the QTL (phenotype) and the flanking markers (see Notes 24 and 25). When the desired region is small enough (preferably containing only a few genes), then additional strategies (see Subheading 3.6.2) can be followed in order to decide which gene in the QTL interval is the QTG.

3.6.2. Other Strategies to Identify the QTG

Complementation Cloning

When the QTL region contains only one gene or up to a few genes, the genomic DNA of the introgressed allele can be cloned and transformed to the recurrent parent in order to complement the phenotype (reinitiate the NIL phenotype in the recurrent background; see Note 26). Detailed information about molecular cloning can be found in (30, 31).

1. Genomic DNA of the accession that provided the introgessed allele can either be isolated from a genomic library if this is available, or otherwise amplified by a PCR (see Note 27).
2. The genomic fragment should be cloned into a binary vector in order to transfer it to the recurrent parent (for transformation of *Arabidopsis thaliana*, see (32)).
3. Grow the primary transformants (T1) and make them homozygous using the selection marker of molecular markers. Grow the homozygous transformants (T2 or T3) and the recurrent parent together, harvest the self-progeny, and analyze their dormancy behavior. If a certain gene induces the expected phenotype in the recurrent background, then that gene can be considered as the QTG.

Screening Existing Mutants: T-DNA Insertion Lines

Screening T-DNA insertion lines is a strategy that can be followed in parallel to the complementation cloning or instead of complementing with several genes. This is targeted to genes in the QTL region with a dormancy/germination phenotype (see Note 28) and was successful for the cloning of *DOG1* (26).

1. Screen the publicly available databases (Table 1) for the presence of T-DNA insertions in the genes that are located in the QTL region. Order the lines and accession in which background they were generated and make them homozygous in cases where they are not already homozygous.
2. Grow the homozygous T-DNA lines together with their background accessions. Harvest the seeds of these lines and analyze their phenotypes. When you identify a line with the desired phenotype, then this is probably the QTG; however, you still have to prove this by complementation cloning (see "Complementation Cloning").

4. Notes

1. Dormancy of *Arabidopsis* seeds is released after the storage of mature seeds in the dry state. This implies that when germination is followed over an interval of time, viable seeds eventually germinate 100%. Therefore, it is important to harvest the seeds when they are just ripe to avoid after-ripening on the mother plant which leads to loss of seed dormancy.
2. For the estimation of the level of seed dormancy, it is important that plants flower at the same time so that seeds that are harvested on the individual genotypes are at the same stage of seed maturation. For assays that require the accurate age of seeds, flowers can be tagged at the day of pollination; siliques can then be harvested at the required stage.
3. It is recommended to use three technical replicates of 50 seeds each (this is common practice in many laboratories when sufficient seeds are available (34)).
4. Seed dormancy can be broken by several agents, such as nitrate, GA, and sugar. So it is important to perform germination assays on water or media that do not contain these agents. Germination experiments are normally performed on filter paper moistened with demineralized water or on plates of water agarose. Sterilization of seeds is not recommended as this may also affect germination, and it is generally not needed when no sugars are added to the medium and the experiment is limited in time (e.g., the 7 days mentioned).
5. Germination tests can also be performed under other conditions, for example, under conditions that promote or prevent dormancy breakage, depending on what one intends to study. For example, conditions like germination at lower or higher temperatures, under different light regimes, or after various seed treatments (such as moist chilling or the addition of nitrate, GA, or ABA) can be studied.
6. Depending on the expected level of dormancy, the schedule can be as follows: 1–3–6–12–18 weeks after seed harvest for very dormant lines and 0–1–2–3 weeks after seed harvest for nondormant lines.
7. Curves of germination percentage as a function of the time of seed dry storage provide the kinetics of seed dormancy of a genotype. Standard curve regression can be performed to fit a logistic curve (Fig. 2) to the individual sets of replicated germination data for each RIL and parents. From the equation of the logistic function, two parameters can be extracted. These include DSDS50; M and the RDR; B.

$$Y = \frac{100}{1 + e^{-B(X-M)}}$$

Fig. 2. Equation of the logistic function. The lower asymptote and upper asymptote, A and C, are constrained to 0 and 100%, respectively, while the slope, B, and inflexion point, M, were estimated. M gives the *d*ays of *s*eed *d*ry *s*torage after which 50% of the seeds germinate (DSDS50). B gives the *rate of dormancy release* (RDR).

8. Currently, there are more than 1,000 different natural accessions of *Arabidopsis* publicly available in stock centers (see Table 1). To estimate the amount of variation that exists within the species for the trait(s) of interest, one should grow as many accessions as possible. However, to reduce efficiently the amount of work, one might select a limited number of accessions that can be chosen according to different criteria. These include: (a) the more than 60 parental accessions of the current permanent mapping populations (http://www.inra.fr/internet/Produits/vast/RILs.htm) or the reference collection of 96 accessions used for linkage disequilibrium (LD) studies (http://www.naturalvariation.org/); (b) a set of accessions chosen on the basis of nucleotide diversity, such as the 8–48 core collections established based on DNA sequence data (35); and (c) a partial collection selected according to geographical and ecological information presumed to be relevant for the trait(s) of interest (http://www.naturalvariation.org/hapmap).
9. Different environments can either be different growing conditions of the mother plants (i.e., temperatures, light conditions, and/or regimes) or different conditions during the germination experiments (see Note 5).
10. To reduce the length of the experiment in more dormant accessions, germination assays can be performed at lower temperatures. Conversely, when the overall level of dormancy is low, germination assays can be performed at higher temperatures (36).
11. The choice of parents for QTL analysis is mostly based on their large phenotypic differences, ideally corresponding to the extremes from the phenotypic distribution of the accessions studied. However, it is also advisable to take one of the common laboratory strains, such as Columbia, L*er*, or Wassilewskija, as one of the parents because more experimental resources are available. In addition, a reference genetic background is provided in this way which allows comparison of the effects of QTL and mutant alleles. To accelerate the construction and analysis of QTL mapping populations, late flowering or very dormant accessions should be avoided as parents unless these are the traits of interest.

12. If accessions carry alleles with opposite effects at several QTL affecting a trait, their joint effects may result in a similar phenotype, and consequently this genetic variation goes undetected when studying the accessions directly. For this reason, the analysis of segregating populations may reveal the presence of individuals with phenotypes outside the range of the parental accessions (transgressive genotypes).
13. In order to obtain unbiased estimates of means and variances, plants should be grown in random designs, such as a fully randomized design or a randomized block design. Growing the plants of the same genotype close to each other might lead to an underestimation of the environmental variance effects and an overestimation of the genetic variance and heritability. For the analysis of seed dormancy, which is largely affected by maternal effects, the environmental influence should be reduced by growing all maternal materials within the same environment.
14. Germination percentages might require transformation, such as angular transformation (=arcsin$\sqrt{}$) or probit transformation, to improve normality before the data is used for QTL analyses.
15. Interactions among QTL identified can be tested by ANOVA using the corresponding markers as random factors.
16. The size of the F2 population that is required to remove other segregation loci in the recurrent background number depends on the number of loci that have to be fixed for one of the two alleles. One can estimate the frequency of the required genotype and then generate a population size, where one expects three plants of the desired genotype to be sure that one does not miss this genotype by chance ($P < 0.05$).
17. Sometimes, another generation is required to "clean up" all regions. Often, it is enough to grow a pre-NIL that contains still some heterozygous regions that segregate in this subsequent generation.
18. Seed dormancy is influenced strongly by the environment; therefore, it is necessary to check dormancy phenotypes of the progeny of F3 plants (F4 seeds). By testing 12 F3 plants, these plants together estimate the F2 phenotype which is more accurate than the result of a single F2 plant. With 12 plants, all genotypes that segregate in the progeny of a heterozygote should be present with $P < 0.05$.
19. HIFs are not to be compared with the reference parental genotype, but with one another within the descendants (family) of the chosen RIL.
20. For the fine mapping of NILs, crossing of the NIL to its recurrent parent is usually enough, as polymorphisms occur due to the different alleles at the position of the introgression. Crossing

to any other accession is possible; however, it creates more genetic variation and this in turn could add complexity as far as determining the phenotype is concerned.

21. The size of the F2 population that is required to fine map the QTL depends on the size of the introgression. In a population of 1,000 F2 plants, one has 2,000 gametes represented. With 1 cM being on average 250 Kbp (37), one recombinant representing 0.05 cM should on average be found for every 12.5 Kbp (a region which might represent a few genes). However, recombination is a chance event and its frequency is also not equally distributed over the chromosomes (38).
22. The distance of the markers to the gene can be calculated; therefore, the number of recombinants has to be divided by the total number of gametes; when multiplied by 100, this is the distance in cM.
23. It might be useful to genotype the F3 plants because recombinants often are homozygous for one marker and heterozygous for the other. The genotype of the homozygous recombinant is most informative. This can also be done only for the recombinants with crossovers close to the locus.
24. In Table 1, several Web sites are shown, where molecular markers can be found or where polymorphisms can be found in order to develop new markers. However, often there is not too much information on polymorphisms in very narrow regions. To be able to develop molecular markers close to the gene of interest, one can sequence that region for both accessions to identify polymorphisms or use a more "old-fashion" technique to develop a CAPS marker by testing the PCR product with several restriction enzymes to identify a polymorphism between both accessions.
25. When there are no recombinants between phenotype and genotype anymore and there are still many genes (>20) located in the fine-mapped region, it might be necessary to increase the F2 population and screen for more recombinants.
26. Keep in mind that complementation is only informative if the dominant allele is transformed to the recessive parent. This implies that if a loss of function mutant is available, this is a useful recipient.
27. Use the coding region, including the 3′ and 5′ (promoter) regions of the gene, for the genomic complementation. A length of 1–1.5 Kb of the promoter sequence and 500–1,000 bp of 3′ region is most of the times sufficient.
28. It is not always clear which phenotype to expect in a knockout line. In addition, the line that has been used for the mutagenesis (in most cases, Col-0) can, for example, have a loss-of-function allele which means that a T-DNA insertion in this gene would not result in a phenotype.

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Chapter 12

Identification of Seed Dormancy Mutants by Activation Tagging

Tiehan Zhao, Ying Zeng, and Allison R. Kermode

Abstract

Activation tagging is an important tool for gene discovery in plants. This method utilizes a T-DNA sequence that contains four tandem copies of the cauliflower mosaic virus *35S* enhancer sequence or promoters oriented outward to the T-DNA border sequences. These elements enhance the expression of genes neighboring on either side of the randomly integrated T-DNA, resulting in gain-of-function phenotypes. Activation tagging has identified a number of genes, including those fundamental to plant development, such as the floral inducer gene, *FLOWERING LOCUS T*(*FT*). The methods surrounding activation-tagging approaches are described in this chapter. While seeds have generally not been the targets of these methods in the past, activation tagging provides a powerful approach to uncover genes involved in seed dormancy and germination, including those that mediate hormone signal transduction.

Key words: Activation tagging, Gain-of-function mutants, Seed dormancy, Seed germination

1. Introduction

Insertional mutagenesis is one of the most powerful approaches to analyze gene function. The insertion of a T-DNA or a transposable element into the plant genome can create mutants. Conventional insertional mutagenesis usually generates recessive loss-of-function mutations, which can be studied only when the mutated gene is homozygous. Therefore, they are unsuitable for functional analysis of redundant genes. To address this significant obstacle, activation tagging provides an alternative method to knockout strategies by upregulating native gene expression (1). Activation tagging employs a tetrameric cauliflower mosaic virus (CaMV) 35S transcriptional enhancer in a T-DNA or a transposon resulting in ectopic expression or overexpression of nearby genes through

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transcriptional activation; this generates gain-of-function mutations. This is a powerful approach, especially for large gene families with functional redundancy of the individual members. Furthermore, recent research indicates that T-DNA is preferentially inserted in the 5′- and 3′-regulatory regions of genes (2). In *Arabidopsis thaliana*, T-DNA insertion into the regulatory regions of genes occurs 2–3 times more frequently than insertion into the coding regions of genes (2). At the gene level, T-DNA integration events show a statistically significant preference for insertion in the promoter region (3). Therefore, a promoter-activation tagging strategy (in which a promoter is oriented "outward" from a transposon or a T-DNA) complements enhancer-activation tagging methods. One advantage of promoter-activation tagging is that it overexpresses only the one adjacent gene, thus overcoming the disadvantage of a spectrum of genes that are responsive to transcriptional enhancement by 35S enhancers. In addition, a modification of promoter-activation tagging, which can be used to mis-express genes during specific phases of the life cycle or in specific tissues, is an estradiol-inducible promoter-activation tagging system. This system has been used to identify genes involved in plant phytohormone signaling (4, 5); the system can be manipulated so that gene activation can be temporally and spatially controlled.

Activation tagging has proven especially useful in the isolation of genes that play roles in plant growth and development (6). It has further led to the discovery of an unforeseen class of gene regulation, involving microRNAs, which plays a fundamental role in plant development as well as in modulating seedling stress responses (7, 8). A recent study employing activation tagging has shed new light on seed germination research (9). Seeds of the three dominant *cold temperature germination* (*ctg*) mutants, which germinate faster than wild-type seeds at 10°C, were recovered from screening activation-tagged lines. Although the use of enhancer- and promoter-activation tagging has not yet been extensively applied in seed dormancy research, it is likely that these approaches will represent a powerful strategy to discover genes directly involved in seed dormancy and/or germination. In this chapter, we detail the methods associated with this approach and further discuss their potential for uncovering mutants affected in seed dormancy or early germination.

2. Materials

2.1. Generation of Transgenic Arabidopsis Plants Expressing Activation-Tagging Vectors

1. Activation-tagging vectors: pATG101 and pATG201 (Fig. 1a, b).
2. *Arabidopsis* seed: Columbia-0 or other ecotypes, such as Cape Verde Islands (Cvi).
3. *Agrobacterium* strain(s): GV3101 (10) or LBA 4404 (11).

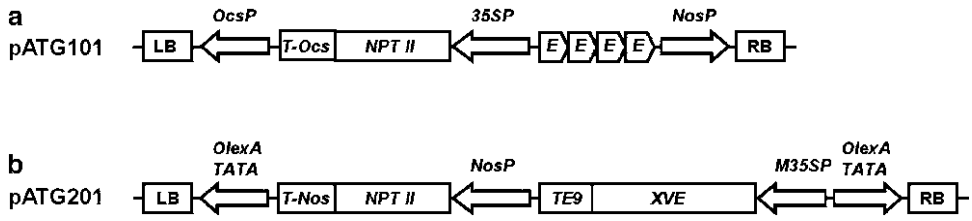

Fig. 1. A schematic illustration of the activation-tagging vectors. (**a**) The activation vector pATG101 contains a tetrameric CaMV 35S transcriptional enhancer (E). A *nopaline synthase* promoter (*NosP*) and an *octopine synthase* promoter (*OcsP*) are oriented outward to the left border (LB) and right border (RB), respectively. A transcriptional unit contains the CaMV 35S promoter, a kanamycin resistance gene (*NPTII*), and a *Nos* terminator (*TNos*) for plant selection. T. Zhao et al., unpublished. (**b**) The bidirectional chemically inducible activation vector pATG201 contains a CaMV minimal promoter (*M35SP*), an *XVE* transcription factor gene, and a ribulose diphosphate carboxylase E9 terminator (*TE9*). The chimeric *XVE* protein consists of the DNA-binding domain of the bacterial repressor LEXA (*X*), the acidic transactivation domain of VP16 (*V*), and the regulatory domain of the human estrogen receptor (*E*). The two *XVE*-responsive promoters – each comprising eight copies of the *LexA* operator sequence fused to the basal CaMV *35S* promoter sequence (*OlexA-TATA*) – are oriented outward to the left border and right border, respectively. A second transcriptional cassette contains a *Nos* promoter (*PNos*), an *NPTII* coding region, and a *TNos* for plant selection. T. Zhao et al., unpublished.

4. Growth chamber (long-day conditions: 16-h photoperiod), 21°C.
5. LB media (solid and liquid): Add 10 g of bacto-peptone, 5 g of yeast extract, 10 g of NaCl, and 15 g of agar (for solid medium) per liter. Adjust pH to 7.4, and autoclave for 20 min.
6. Selection plates: Half-strength Murashige and Skoog (MS) medium (PhytoTechnology Laboratories, Shawnee Mission, KS), 1% of sucrose, adjust pH to 5.8, 2 g/L of Gellan Gum Powder (PhytoTechnology Laboratories). After autoclaving, cool medium to approximately 50°C and then add the selectable marker agent (e.g., kanamycin; final concentration of 50 mg/L).
7. Silwet L-77 (Lehle Seeds).
8. Sucrose solution: 5% (w/v).
9. Antibiotics (e.g., for *Agrobacterium* selection: rifampicin 25 mg/L, gentamicin 25 mg/L, and spectinomycin 100 mg/L).
10. Ethanol: 70% (v/v).
11. Bleach solution: 40% commercial bleach (2.4% hypochlorite), 0.05% Tween-100 (v/v).

2.2. Screening of Seed Mutant Phenotypes

1. Stock solution of (+)-abscisic acid (ABA) (PhytoTechnology Laboratories), 5 mM: Add the appropriate weight of ABA to make a 5 mM (1.32 mg/mL) stock solution and store at –20°C until use. ABA does not dissolve in water directly, so first dissolve

it in a few drops of ethanol, methanol, or DMSO, and then add ddH_2O to bring up to the desired concentration. Filter-sterilize and aliquot prior to storage at –20°C, and use a vessel that protects the ABA from light.

2. MS plates containing (+)-ABA (5–100 μM): Prepare with half-strength MS salts and 1% sucrose, pH 5.8. 2 g/L Gellan Gum Powder (PhytoTechnology Laboratories). After autoclaving, cool medium to approximately 50°C and add ABA to a final concentration of 5 μM, 10 μM, and other desired concentrations up to 100 μM.
3. Chemical inducer 17-β-estradiol: 20 mM 17-β-estradiol (Sigma-Aldrich) in 70% ethanol or 100% DMSO. Store at –20°C.

2.3. Isolation of Genomic Sequences Flanking T-DNA Insertions

1. Qiagen DNeasy Plant Mini Kit for extraction of genomic DNA from seedlings.
2. T4 ligase (Invitrogen).
3. Restriction enzymes (New England BioLabs or Fermentas).
4. dNTP stock (Fermentas): dNTP mixture comprising 2.5 mM each of dATP, dCTP, dGTP, and dTTP. Store at –20°C.
5. DNA polymerases: *Taq* DNA polymerase (Fermentas), Biolase Red DNA Polymerase (Bioline), Expand Long Template enzyme mix (Roach), Phusion DNA Polymerase (New England BioLabs).
6. PCR DNA and Gel Band Purification Kit (GE Healthcare) for isolation of PCR fragments.
7. Adaptors (AD1 and AD2):

   AD1:5′-CTAATACGACTCACTATAGGGCACGCGTGGTC GACGGCCCGGGCTGGT-3′

   AD2: 5′-P-ACCAGCCC-NH2-3′
8. T-DNA primers:

   NosP1: 5′-GAACTGACAGAACCGCAACGTTG-3′

   NosP2: 5′-AGTCGCCTAAGGTCACTATCAGC-3′

   OcsP1: 5′-ACTCATCCACATTGATGGTAGGC-3′

   OcsP2: 5′-AGGTGTGGCCTCAAGGATAACG-3′

   LexA1: 5′-GTACTGTACATATAACCACTGG-3′

   LexA2: 5′-ACTGGTTTTATATACAGCAGTACTG-3′
9. Adaptor primers:

   ADP1: 5′-CTAATACGACTCACTATAGGGC-3′

   ADP2: 5′-ACTCACTATAGGGCACGCGTGGT-3′

3. Methods

Activation tagging is a powerful mutagenesis strategy that generates dominant, gain-of-function mutations resulting from gene overexpression by randomly inserting regulatory sequences throughout the genome. The activation T-DNA-tagging vector pATG101 utilizes a "cassette" comprising four tandem copies of the CaMV *35S* enhancer sequence and the promoters of the *Agrobacterium octopine synthase* (*Ocs*) and *nopaline synthase* (*Nos*) genes that are placed adjacent to the left and right T-DNA borders (Fig. 1a). These elements enhance the expression of neighboring genes on either side of the randomly integrated T-DNA tag, resulting in gain-of-function phenotypes. However, using this activation tagging approach, a stronger seed dormancy phenotype may be missed due to the fact that deep seed dormancy arising from constitutive ectopic expression of a given gene would be indistinguishable from nontransgenic seeds that are subjected to selection conditions (presence of the selectable marker agent in the culture medium). To overcome this and related problems, a bidirectional chemically inducible promoter-activation tagging vector pATG201 has been developed (Fig. 1b). Promoters that are inducible by 17-β-estradiol are placed adjacent to the T-DNA right and left border sequences to drive the conditional expression of neighboring genes. The system can be used to generate conditional mutant alleles to uncover deep seed dormancy and early lethal mutants. Since the sequence of the insertion vector as a molecular tag is known, the overexpressed genes can easily be isolated using standard molecular biological techniques.

3.1. Generation of Transgenic Arabidopsis Plants Expressing Activation-Tagging Vectors

This protocol was adapted from Clough (12) and Zhang (13).

1. Grow and prepare the *Agrobacterium* strain (GV3101) harboring an activation-tagging vector by inoculating a single *Agrobacterium* colony into 5-mL liquid LB medium with the appropriate antibiotics for binary vector selection (rifampicin 25 mg/L, gentamicin 25 mg/L, and spectinomycin 100 mg/L). Incubate the cultures at 28°C with shaking (250 rpm) for 2 days (see Note 1).
2. Inoculate this feeder culture by adding 200 mL liquid LB containing the appropriate antibiotics noted above and grow the culture at 28°C for 24 h to reach an OD > 1 (at 600 nm).
3. Collect *Agrobacterium* culture by centrifugation at 4,000 × *g* for 10 min at room temperature, and resuspend cell pellets in 500 mL of freshly made 5% (w/v) sucrose solution.
4. Add Silwet L-77 to a concentration of 0.02% (v/v) (100 µL per 500 mL of sucrose solution) and mix well immediately

before conducting step 5. Transfer the *Agrobacterium* cell suspension to a 500-mL beaker.

5. Invert a pot containing an *Arabidopsis* plant and dip the aerial parts of the plant into the *Agrobacterium* cell suspension for 30 s with gentle agitation. Remove dipped plants from the solution and drain the treated plants for 3–5 s.
6. Place the pots in a plastic tray and cover with a transparent plastic cover for 16–24 h to maintain high humidity and return the treated plants back to normal growing conditions (i.e., a growth chamber maintained at 21°C with a 16-h photoperiod).
7. Collect dry seeds after 4 weeks.
8. Surface-sterilize seeds by soaking them in 70% (v/v) ethanol for 1 min and then in 40% bleach (2.4% hypochlorite) containing 0.05% (v/v) Tween-100 for 10 min. Wash the seeds three times with sterile water, and then resuspend the sterilized seeds in sterile 0.1% agarose and spread onto selection plates (see Subheading 2.1, item 6). Allow plates to dry a little.
9. Moist-chill the seeds by placing the plates at 4°C for 3–4 days or directly transfer the plates to a growth chamber or tissue culture incubator maintained at 21°C with a 16-h photoperiod.
10. After 7–10 days, putative transformants are readily distinguished as seedlings with green cotyledons, true leaves, and roots that extend into the selective medium.
11. Transfer the transgenic seedlings to pots containing soil. After growth of plants, flowering, and seed maturation, harvest the mature dry transgenic seeds and mix 100 independent lines as one pool for further mutant screening.

3.2. Screening of Seed Mutant Phenotypes

3.2.1. Screening of ABA-Insensitive Mutants

ABA has an established role in promoting seed maturation processes and in the inception and maintenance of seed dormancy. Seeds of mutants affected in ABA biosynthesis or response usually show altered seed dormancy. The protocol described here uses the selection of ABA-insensitive mutants as an example. Technically, we have not targeted dormancy per se, as the criterion for selection is a relative insensitivity of seeds to ABA as far as an inhibition of their early postgerminative growth is concerned. Similar approaches can be developed for screening mutants affected in dormancy (noted below in Subheading 3.2.2) or in signal transduction pathways of other hormones.

1. Prepare 125 plates containing half-strength MS salts and 1% sucrose with 5 or 10 μM ABA.
2. Weigh a total of 6.0 g of seeds (see Note 2) from all of the pools obtained (see above, Subheading 3.1, step 11). Surface-sterilize seeds with 40% bleach (2.4% hypochlorite) for 10 min using a

500-mL flask. Wash the seeds in sterilized water three times for 5 min each.

3. Resuspend seeds in 375 mL of 0.1% agarose solution. This results a "concentration" of ~16 mg seeds/mL.
4. Distribute 3 mL of seeds (~2,000 seeds) on each plate. Swirl plates to spread seeds evenly on the entire surface.
5. Maintain plates at 4°C for 4 days.
6. Transfer plates to a growth chamber maintained at 21°C and long-day conditions (16-h photoperiod) for germination.
7. The majority of seeds germinate (undergo radicle emergence), but are unable to continue postgerminative growth (seedling development). Identify the seeds/seedlings that are able to elongate roots, develop true leaves, and continue seedling growth. They are putative ABA-insensitive mutants (Fig. 2a).
8. Transfer the putative candidate seedlings to soil and grow them normally in a growth chamber under long-day conditions. Observe carefully and record any abnormal phenotypes during plant growth.
9. Following flowering, harvest immature and mature seeds for further analyses (see Note 3). Insensitivity to ABA of the identified candidates should be first verified before conducting other analyses (Fig. 2b).

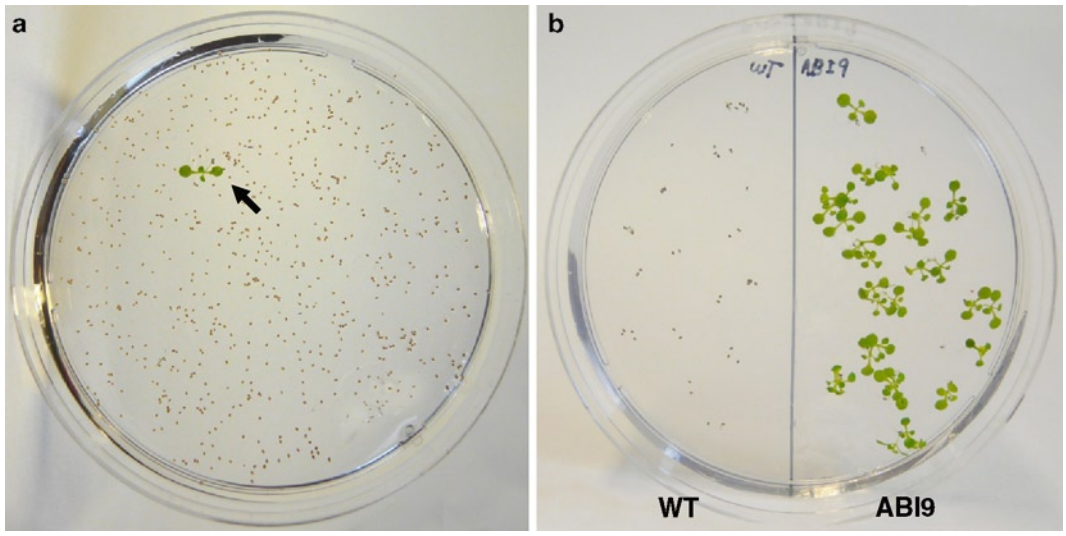

Fig. 2. Screening and verification of ABA-insensitive mutants. (**a**) Screening of T-DNA-transformed seeds on half-strength MS medium with 5 μM of (+)-ABA. Only the putative ABA-insensitive mutant (indicated by an *arrowhead*) was able to complete germination and continue postgerminative growth. (**b**) To verify the property of ABA insensitivity, seeds of the putative candidate plant were sown on 5 μM ABA plates (on the *right* of the plate) for germination and growth assessment using WT (Col-0) as the control (*left*). Picture shows their performance after a 10-day incubation in a growth chamber at 21°C, 16-h light period.

3.2.2. Screening of Mutants in Seed Dormancy

Seeds of mutants affecting dormancy may exhibit either an enhancement of dormancy or reduced dormancy. The protocol described here is for selection of seeds with reduced dormancy.

1. Combine each ten pools (see Subheading 3.1, step 11) of seeds into large pools for easier handling. Weigh 240 mg of seeds from each large pool (about ten seeds from each plant). Keep the remaining seeds for later use to screen for other mutants.
2. Seeds are surface-sterilized on the day of harvest and resuspended in 0.1% of agarose (see Subheading 3.2.1).
3. Plate 3 mL of seed solution on each solid plate containing only half-strength MS salts.
4. Incubate plates under germination conditions (16-h photoperiod, 21°C) without any moist-chilling treatment.
5. Examine plates daily. Seeds that germinate very early are selected as putative mutants for reduced seed dormancy (see Note 4).
6. Grow the mutant candidate plants normally and harvest seeds from each plant individually.
7. On the same day of harvest, put about 100 seeds from each plant on half-strength MS plates (containing 1% of sucrose) and incubate under germination conditions to confirm the phenotype of early germination in the absence of moist chilling.
8. Confirmed mutant plants are used for gene identification and further analysis.

3.2.3. Screening for Mutants with Reduced Seed Dormancy Using 17-β-Estradiol Treatment

1. Harvest fresh T2 seeds transgenic for pATG201 and mix seeds in pools each containing about 100 transgenic lines. Place seeds on plates containing half-strength MS salts supplemented with 10 μM 17-β-estradiol and 50 mg kanamycin. As a control, place seeds from the same pool on plates containing half-strength MS salts with 50 mg kanamycin only.
2. Place control/experimental plates in germination conditions (16-h photoperiod; 21°C) to monitor germination in the absence of moist chilling (see Note 5). Examine plates daily to identify the early germinating seeds (see Note 4).
3. Transfer the seedlings into soil and harvest the next-generation seeds.
4. Repeat the screen as above to confirm the early germination phenotype.

3.2.4. Screening for Mutants with Enhanced Seed Dormancy (Delayed Germination) Using 17-β-Estradiol Treatment

1. Plate freshly harvested T2 seeds as in step 1 of Subheading 3.2.3 above.
2. Moist-chill the seeds at 4°C for 4 days. Then, transfer the plates into a growth chamber maintained at 21°C (16-h photoperiod).

3. Identify seeds that show delayed germination or those that have not germinated after 7–10 days and transfer them onto germination plates (containing half-strength MS salts and kanamycin) but no 17-β-estradiol. Maintain plates under germination conditions in growth chamber (16-h photoperiod; 21°C).
4. Transfer recovering seedlings into soil. Harvest the next-generation seeds and repeat the screen as above to confirm the deep dormancy mutant phenotype.

3.3. Isolation of Genomic Sequences Flanking T-DNA Insertions

The PCR-based methods, such as thermal asymmetric interlaced (TAIL) PCR, inverse PCR (IPCR), and adapter-ligation PCR, have been developed for isolation of genome DNA flanking the T-DNA insertions.

3.3.1. TAIL PCR

TAIL PCR uses specific primers designed from the known sequence of T-DNA and arbitrary primers that may bind somewhere on the unknown genomic DNA. One or several fragments can be amplified after a few steps of complex PCR cycles, which involve alternating high- and low-stringent conditions. The following protocol is adapted from (14), with our modifications. We use three gene-specific primers with relatively long sequences, each having a T_m of ~72°C and an arbitrary primer referred to as AD2 in (14). The sequence is: 5′-NGTCGA(G/C)(A/T)GANA(A/T)GAA-3′ (128-fold degeneracy, average T_m = 46.6°C).

PCRs (containing 50 μL) are set up with 5 μL 10× PCR buffer, 200 μM each of dNTPs, 2 μM of each primer, DNA template (50 ng of genomic DNA for the initial cycle or 1 μL of a tenfold dilution of prior PCR products for later cycles), and 0.8 μL of Phusion DNA polymerase. The PCR thermal cycle settings are outlined in Table 1.

1. For initial amplification, add only gene-specific primer 1 (GSP1, the outermost primer) to the reaction mix and carry out ten cycles of amplification to enrich gene-specific templates (see Note 6).
2. After the initial amplification, add arbitrary degenerate primer AD2 to the reaction mix and an additional 0.2 μL of Phusion DNA polymerase, and proceed to primary reaction cycles.
3. Dilute 10× of the primary PCR products and use 1 μL for secondary PCR. Primers, GSP2 and AD2, are used in secondary reactions.
4. Dilute 10× of the secondary PCR products and use 1 μL for tertiary PCR. Primers, GSP3 and AD2, are used in tertiary reactions.
5. Load secondary and tertiary PCR products on a 1.2% agarose gel and separate them by electrophoresis.

Table 1
TAIL PCR cycle settings

Reaction	Cycle number	Program settings
Initial	1	98°C, 2 min
	10	98°C, 15 s; 72°C, 2 min (see Note 6)
	1	8°C, hold
Primary	1	98°C, 1 min
	1	98°C, 15 s; 25°C, 3 min, ramping to 72°C in 3 min, 72°C, 1 min 30 s
	15	98°C, 15 s; 72°C, 1 min 30 s
		98°C, 15 s; 72°C, 1 min 30 s
		98°C, 15 s; 44°C, 20 s; 72°C, 1 min 30 s
	1	72°C, 5 min; 8°C, hold
Secondary	1	98°C, 1 min
	12	98°C, 15 s; 72°C, 1 min 30 s
		98°C, 15 s; 72°C, 1 min 30 s
		98°C, 15 s; 44°C, 20 s; 72°C, 1 min 30 s
	1	72°C, 5 min; 8°C, hold
Tertiary	1	98°C, 1 min
	25	98°C, 15 s; 72°C, 1 min 30 s
		98°C, 15 s; 72°C, 1 min 30 s
		98°C, 15 s; 37°C, 20 s; 72°C, 1 min 30 s
	1	72°C, 5 min; 8°C, hold

6. Cut putative positive DNA bands from the gel and purify DNA with GFX PCR DNA and Gel Band Purification Kit (see Note 7).
7. Directly sequence the purified DNA using the GSP2 or GSP3 primer. If the sequence contains the corresponding T-DNA fragment plus an extra sequence, then it is a genomic sequence flanking the T-DNA and its identity can be searched by the online BLAST programs from the National Center for Biotechnology Information (NCBI) or The Arabidopsis Information Resource (TAIR).

3.3.2. Inverse PCR

A modified version of IPCR (described in (15)) can also be used to isolate the genomic DNA flanking T-DNA insertions.

1. Digest 500–1,000 ng of genomic DNA with an appropriate restriction enzyme, which cleaves within the T-DNA in a 100-μL volume for 3 h (see Note 8).
2. Purify the digested DNA using the GFX PCR DNA and Gel Band Purification Kit.

3. Self-ligate the DNA in a 100-μL reaction volume using 5 U of T4 ligase at 4° or 16°C overnight.
4. Purify the ligated DNA using the GFX PCR DNA and Gel Band Purification Kit.
5. Design two nested inward PCR primers: the inward primers face the restriction enzyme cleavage site within the T-DNA (see Note 8).
6. In the first round of PCR, use 2.5 μL (10 μM) of outward primer (NosP1, OcsP1, or LexA1) and 2.5 μL (10 μM) of inward primer 1, 5 μL of buffer, 4 μL of dNTP mix (2.5 mM), 5 μL of ligated DNA (50 ng), and 1 U of Biolase Red DNA Polymerase (Bioline) in a 50-μL reaction volume. Denature at 94°C for 2 min and then perform 30 cycles of amplification (30 cycles of 94°C, 1 min; 55–60°C, 1 min; 72°C, 3 min), and a final 10 min at 72°C.
7. Run out 5 μL of PCR products on a 1% agarose gel (see Note 9).
8. In the second round of PCR, use 2.5 μL (10 μM) nested outward primer (NosP2, OcsP2, or LexA2) and 2.5 μL (10 μM) nested inward primer 2, and 1 μL of 10- to 50-fold diluted first-round PCR products. Conduct amplification under the same conditions as noted for the first-round PCR, but conduct 35 cycles (see Note 10).
9. Run out 5 μL of PCR products on a 1% agarose gel.
10. The IPCR products are gel-purified using the GFX PCR DNA and Gel Band Purification Kit and then sequenced.

3.3.3. Adaptor-Ligation PCR

A modified adaptor-ligation PCR procedure (16) can be used to amplify genomic DNA flanking the T-DNA insertion.

1. To anneal the adaptors, mix 25 mM of long and short adaptors (see Subheading 2.3) in any PCR buffer without dNTPs in a 100-μL volume for 5 min at 94°C. Follow by gradual cooling at room temperature.
2. Digest 1 μg of genomic DNA for 3–6 h in a 100-μL volume with 10 U of a restriction enzyme; use those that are 6-bp blunt-end cutters, such as *Eco*RV or *Ssp*I.
3. Purify the digested DNA with the GFX PCR DNA and Gel Band Purification Kit.
4. Ligate the digested DNA to 1 μL (25 mM) of annealed adaptors and 5 U of T4 DNA ligase in a 100 μL volume at 16°C overnight.
5. Purify the ligated DNA using the GFX PCR DNA and Gel Band Purification Kit. Elute the ligated DNA with 100 μL of elution buffer.

6. Design two nested T-DNA-specific PCR primers facing the T-DNA end (left or right border) (see Subheading 2.3).
7. In the first round of PCR, use 10 μL of the ligated DNA, 5 μL of 10× PCR buffer, 4 μL of dNTP mix (2.5 mM), 2.5 μL (10 μM) of AP1, 2.5 μL (10 μM) of T-DNA primer (NosP1, OcsP1, or LexA1), and 0.75 μL of Expand Long Template enzyme mix (Roach) in a 50-μL reaction volume. Assemble the reaction mixes as recommended by the supplier. Perform 30 cycles of PCR (94°C, 2 min; 94°C, 15 s; 65°C, 30 s; 68°C, 3–5 min). Final elongation is for 7 min at 68°C.
8. In the secondary round of PCR, use 1 μL of 10- to 50-fold diluted first-round PCR products. Perform secondary PCR under the same conditions as the first-round PCR, but using AP2, nested T-DNA primers (NosP2, OcsP2, or LexA2) and 35 cycles (see Note 11).
9. Use 5 μL of the PCR mix to detect the product on a 0.8% agarose gel. Isolate distinct bands from the rest of the PCR mix using the GFX PCR DNA and Gel Band Purification Kit. Clone the band of interest and sequence.

3.4. Confirmation of Mutant Phenotype

Once a mutant has been isolated and the specific tagged gene (putatively causing the phenotype) has been cloned, further work can be undertaken to characterize the function of the gene and confirm the basis of the mutant phenotype. These studies can include conducting the following: (1) analysis of the activated gene(s) by quantitative RT-PCR or Northern blots using physiologically relevant stages of seed/seedling development. (2) Cloning the cDNA of the activated gene into a T-DNA binary vector (17, 18) to overexpress it under the control of a CaMV 35S promoter. Wild-type plants can then be transformed by the floral dip transformation. Confirm if the overexpressed gene causes the dominant phenotype. (3) Cloning the cDNA of the activated gene into an estradiol-inducible expression vector (18, 19) to conditionally control the expression of the activated gene in wild-type plants. Investigate if the transformants can reproduce the dominant mutant phenotype. (4) Use various biochemical/molecular biological approaches to functionally analyze and characterize the genes (see other chapters).

4. Notes

1. When stored at 4°C, tetrameric CaMV 35S enhancers tend to be unstable and there can be a loss of copies of these sequences in *Agrobacterium tumefaciens*. Therefore, always inoculate the cultures from plates that have been streaked from −80°C stocks

and check the number of copies of CaMV 35S enhancers by PCR using flanking oligonucleotide primers.

2. One hundred seeds weigh ~2.4 mg. If one considers 25,000 plants and the use of ten seeds from each plant, there will be 250,000 seeds that roughly weigh 6 g in total.
3. Carefully observe seeds of each plant during their maturation. If seeds are unable to undergo desiccation during their late maturation and show wrinkled green phenotypes, the more immature seeds of the same plant need to be harvested and germinated before normal maturation drying to keep the line alive.
4. To qualify as candidates, seeds should germinate within 24 h and the percentage of seeds that germinate early should comprise about 0.1% or less of the total population. If there are a large number of seeds that germinate around the same time, they should not be considered mutant candidates.
5. 17-β-estradiol is light-sensitive and its activity slowly declines in a light intensity-dependent fashion.
6. We find that this initial ten cycles of amplification with only GSP1 primer are very useful in obtaining a good quantity of gene-specific products in later cycles because they greatly increase the copy number of gene-specific templates.
7. The putative candidate band should have a size of at least 200 bp larger than the predicted T-DNA fragment that would be amplified by the specific primer.
8. Use a 4- or 5-bp cutter restriction enzyme (e.g., AluI, MseI, BstyI, and HinfI) that does not cut the sequence between the ends of forward and reverse primers, but that cuts the length of the DNA that must be available to PCR.
9. Run out 5 μL of first-round PCR products; a good smear or banding pattern should be observed.
10. If no PCR product is obtained from the second round of PCR, try to use other restriction enzymes to digest the genomic DNA.
11. If there are nonspecific PCR products from the second round of PCR, dilute the PCR products from the first round of PCR or increase annealing temperature.

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Chapter 13

Isolation of MicroRNAs that Regulate Seed Dormancy and Germination

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Abstract

MicroRNAs (miRNAs) play an important role in gene regulation in many plant tissues and organs during various developmental stages. Previous studies have suggested the importance of gene regulation by miRNA in seeds. Characterizing the expression of miRNAs and their target genes in dormant and germinating seeds helps to gain a better understanding of the regulatory role of miRNAs during seed dormancy and germination. This can be achieved by implementing a simple miRNA extraction method using fractionation with isopropanol and Northern blot analysis using nonradioactive miRNA probes. Functional analysis of miRNA target genes potentially associated with seed dormancy and germination can be examined using mutant seeds in which specific miRNAs are deregulated by introducing silent mutations in the miRNA target sites of these genes.

Key words: miRNA, Seeds, Dormancy, Germination, Gene expression

1. Introduction

MicroRNA (miRNA) is a single-stranded untranslated RNA of approximately 21 nucleotides, which downregulates gene expression through posttranscriptional cleavage of mRNA or translational repression (1, 2). Since the discovery of miRNA in plants, different miRNAs have been shown to be important during various developmental stages of plants, such as floral induction (3, 4) and development (5, 6), male and female sterility (6, 7), lateral root development (8), auxin signaling (9–11), and RNA metabolism (12, 13). As evident from the literature, miRNA plays a crucial role in the major events during plant development through its involvement in gene regulatory networks mainly by targeting transcription factors. Therefore, modulating the levels of specific miRNAs is necessary for normal plant development (14, 15). Small RNA-degrading

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nucleases (SDNs) help maintain appropriate levels of miRNAs by eliminating excess mature miRNAs (16).

In *Arabidopsis*, 21 miRNA families have been shown to regulate 95 genes and 65 of the target genes (68%) encode transcription factors, suggesting a role for miRNAs at the core of gene regulatory networks (1). miRNAs also play a role in gene expression in seeds and seedlings. The ability of seeds to remain dormant or germinate at appropriate developmental stages and under different environmental conditions is crucial for the survival of plant species. The process of dormancy termination is initiated through the action of hormones and other signaling molecules, which may then activate appropriate signaling cascades leading to radicle emergence and seedling growth. Such a signaling system is likely to be controlled by changes in the expression of important regulatory genes whose products are required to stimulate or inhibit the initiation of germination. The involvement of miRNAs in the regulation of seed germination and dormancy has already been demonstrated in *Arabidopsis*. For example, miR159 is involved in seed germination under stress conditions (17) and miR160 is important in the hormonal control of germination and postgermination stages (18). This chapter describes research methods used to identify and characterize miRNA target genes involved in seed dormancy and germination.

2. Materials

2.1. Identification of miRNA-Targeted, Seed Dormancy- and Germination-Associated Genes

Bioinformatic resources

- www.affymetrix.arabidopsis.info
- http://affymetrix.arabidopsis.info/narrays/experimentbrowse.pl
- www.genevestigator.ethz.ch
- http://asrp.cgrb.oregonstate.edu
- http://bar.utoronto.ca
- http://www.arabidopsis.org
- http://www.ncbi.nlm.nih.gov

2.2. Expression Analysis of miRNA in Seeds

2.2.1. Extraction of miRNA

1. TLE buffer (pH 8.2): 0.18 M Tris, 0.09 M LiCl, 4.5 mM EDTA, 1% (w/v) sodium dodecyl sulfate (SDS). Store in refrigerator.
2. β-mercaptoethanol.
3. Phenol (Tris buffer pH 8.0-equilibrated). Store in refrigerator.
4. Chloroform.

5. Phenol:chloroform:isoamylalcohol [25:24:1 (v:v:v)]. Store in refrigerator.
6. Buffer A: 5-mL TLE buffer, 5 μL β-mercaptoethanol, 5 mL phenol, and 1 mL chloroform.
7. 20% (w/v) polyvinylpyrrolidone (PVP). Always prepare fresh.
8. 8 M LiCl. Store in refrigerator.
9. Isopropanol.
10. 4× BPB buffer: 500 μL glycerol, 100 μL of 0.5% (w/v) BPB solution, 50 μL of 1 M Tris–HCl buffer, pH 8.0, 10 μL of 500 mM EDTA and water to a final volume of 1 mL.
11. miRNA loading buffer: 500 μL formamide, 250 μL 4× BPB buffer, and 250 μL water.

2.2.2. Separation and Transfer of miRNA

1. 30% acrylamide solution: 29.2% (w/v) acrylamide and 0.8% (w/v) bis-acrylamide.
2. 5× TBE (pH 8.0): 54 g Tris base, 27.5 g boric acid, 20 mL 0.5 M EDTA (pH 8.0) in deionized water with final volume of 1 L.
3. Urea.
4. Ethanol.
5. Tetramethylethylenediamine (TEMED).
6. 10% (w/v) ammonium persulfate (APS).
7. Positively charged nylon membrane (e.g., Hybond N^+, Amersham Biosciences, Piscataway, NJ).
8. Ethidium bromide 0.0002% (w/v).
9. Extra thick blot paper (e.g., Bio-Rad, Hercules, CA)
10. Transfer system (e.g., Semi Dry Transfer Cell System, Bio-Rad).
11. Pasteur pipettes.

2.2.3. Detection of miRNA (Northern Analysis)

A. Probe Synthesis
   1. mirVana™ miRNA Probe Construction Kit (Ambion, Austin, TX).
   2. DNA oligos for miRNA probe synthesis (see Subheading 3).
   3. Digoxigenin (DIG) RNA labeling mix (Roche Applied Science, Indianapolis, IN).

B. Hybridization, Washing, and Detection
   1. PerfectHyb ™Plus Hybridization Buffer (Sigma, St. Louis, MO).
   2. Plastic bag (0.11 mm thick; KAPAK, Minneapolis, MN).
   3. Plastic bag sealer (e.g., TEW Electric Heating Equipment Co., Ltd., Taipei, Taiwan).

4. miRNA probes.
5. 2× SSC: 300 mM NaCl, 30 mM sodium citrate, pH 7.0.
6. 10% (w/v) SDS.
7. 1× maleic acid buffer: 100 mM maleic acid, 150 mM NaCl, pH 7.5.
8. Tween-20.
9. Dehydrated nonfat milk.
10. Anti-DIG-alkaline phosphatase (AP) Fab fragments (0.75 U/μL; Roche Applied Science). Store at 4°C.
11. Equilibration buffer: 0.1 M Tris–HCl, 0.1 M NaCl, pH 9.5.
12. Chemiluminescent substrates (e.g., CDP-star Ready for Use, Tropix Inc, Bedford, MA or LumiPhos, Lumigen, Southfield, MI).
13. X-Ray film.

2.3. Expression Analysis of miRNA Target Genes in Seeds

2.3.1. Extraction of mRNA

See Subheading 2.2.1. Extraction of miRNA.

2.3.2. Expression Analysis of mRNA

1. mRNA-specific primers of target genes and housekeeping gene (actin).
2. mRNA isolated from dormant seeds and seeds during early stages of germination (see Subheading 3).
3. Reverse Transcriptase kit.
4. *Taq* DNA polymerase and buffer provided with the enzyme.
5. dNTPs.

2.4. Functional Analysis Using miRNA-Resistant Mutants

2.4.1. Isolation of Genomic Fragments

1. Genomic DNA (gDNA).
2. Gene-specific primers to amplify target genes (see Subheading 3).
3. Proofreading *Taq* DNA polymerase (and buffer provided with the enzyme).
4. dNTPs.
5. Cloning vectors.
6. *Escherichia coli*-competent cells.

2.4.2. Creation of Silent Mutations

1. Plasmid containing genomic fragments of miRNA target genes whose sequences have been confirmed.
2. Primers to create mutations in the miRNA complementary sites in target genes (see Subheading 3).

3. Proofreading *Taq* DNA polymerase (and buffer provided with the enzyme).
4. dNTPs.
5. Restriction enzymes.
6. PCR purification kit.
7. T_4 DNA ligase (and buffer provided with the enzyme).

3. Methods

3.1. Identification of miRNA-Targeted Seed Dormancy- and Germination-Associated Genes

Data from existing microarray databases, such as www.affymetrix.arabidopsis.info, http://affymetrix.arabidopsis.info/narrays/experimentbrowse.pl, and www.genevestigator.ethz.ch, can be mined to identify genes potentially associated with seed dormancy and germination. Genes that are up- or downregulated in dormant or germinating seeds can be identified. After identifying dormancy- or germination-associated genes, the subset of genes that are potentially targeted by miRNAs can be identified by searching miRNA databases, such as http://asrp.cgrb.oregonstate.edu. The expression patterns of the identified genes during seed dormancy and germination can also be confirmed at http://bar.utoronto.ca (G. W. Bassel, unpublished data). After identifying possible dormancy- or germination-associated genes through analysis of *Arabidopsis* microarray databases, information about genes of interest (e.g., gDNA and transcript sequences) can be obtained from databases, such as http://www.arabidopsis.org and http://www.ncbi.nlm.nih.gov.

3.2. Expression Analysis of miRNA in Seeds

Once miRNA-targeted seed dormancy- or germination-associated genes are identified, their expression at various stages of seed imbibition can be examined by Northern blot analysis to verify their association with dormancy or germination.

3.2.1. Extraction of miRNA

1. Harvest germinating *Arabidopsis* seeds (20 mg) and freeze them at −80°C for at least 15 min.
2. Use a mortar and pestle to thoroughly grind frozen seeds in 1 mL of buffer A. Add another mL of buffer A and homogenize tissues thoroughly (see Note 1).
3. Transfer the homogenate to a 2-mL centrifuge tube, vortex, and centrifuge at 10,000 × *g* for 2 min.
4. Transfer upper aqueous phase to a 2-mL tube and add 1 mL of phenol:chloroform:isoamylalcohol (25:24:1, v/v/v) solution. Vortex well and then centrifuge at 10,000 × *g* for 2 min.

5. Transfer upper aqueous phase to a 2-mL tube. Add an equal volume of chloroform, mix well, and then centrifuge at 10,000 × *g* for 2 min.
6. Transfer upper aqueous phase to a 1.5-mL tube. Add 1/8 the supernatant volume of 20% (w/v) freshly prepared PVP solution and mix well by inverting the tube. Then, add 1/3 total solution volume of 8 M LiCl (8 M LiCl:[supernatant + PVP] = 1:3) and mix thoroughly (see Note 2).
7. Keep the tube at –80°C for 10 min.
8. Completely thaw contents of tube and invert tube to mix well.
9. Centrifuge at 10,000 × *g* for 2 min. Transfer the supernatant to a fresh tube without disturbing the pellet (see Note 3).
10. Add isopropanol to bring the final concentration to 40% (v/v) (add 2/3 of the original sample volume of isopropanol; e.g., if supernatant including LiCl is 1,000 μL, then add 666 μL isopropanol). Mix well (see Note 4).
11. Incubate at room temperature for 15 min and then centrifuge at 10,000 × *g* for 2 min.
12. Transfer supernatant to a fresh tube and add isopropanol to bring the final concentration to 50% (v/v) (add 1/2 the amount of isopropanol added in the previous step, i.e., 333 μL). Mix well (see Note 4).
13. Incubate solution at room temperature for 15 min and then centrifuge at 10,000 × *g* for 2 min.
14. Discard the supernatant and wash the pellet with 1 mL of 80% (v/v) ethanol.
15. Centrifuge at 10,000 × *g* for 2 min, decant the supernatant, and dry the pellet.
16. Resuspend the pellet in 10–20 μL of miRNA loading buffer and store at –80°C until use (see Note 4).

3.2.2. Separation and Transfer of miRNA

Before loading miRNA samples on a 17% (w/v) polyacrylamide gel containing 7 M urea, equal loading of miRNA from different seed samples can be examined by loading 1–3 μL of miRNA samples on a 1–1.5% (w/v) agarose gel.

Electrophoresis

1. To prepare a 17% acrylamide gel containing 7 M urea, mix 1.0 mL of 5× TBE buffer, 5.7 mL 30% (w/v) acrylamide/bis-acrylamide solution (37.5:1), 0.5 mL water, and 4.2 g urea in a 50-mL tube. Heat the tube to ~40°C in a water bath to completely dissolve the urea (see Note 5).
2. While the urea is dissolving, prepare gel rig for pouring plates. Clean glass plates and 1.5-mm spacers with ethanol before use (see Note 6).

3. Cool gel mixture to ~30°C. Add 50 μL of 10% (w/v) APS solution and 5 μL TEMED. Invert gently to mix and pour gel. Carefully insert the 1.5-mm-thick comb without trapping air bubbles. Allow the gel to polymerize for 30 min.
4. Assemble electrophoresis system and add running buffer (0.5× TBE).
5. Wash wells with a syringe to remove the precipitated urea, which could decrease band sharpness.
6. Pre-run gel at 180 V for 60 min.
7. Again wash wells with a syringe to remove any residual urea.
8. Load equal amounts of miRNA samples into the bottom of each well.
9. Run gel at 180 V until dye front reaches the end of the gel.
10. Remove gel from apparatus and cut one corner of the gel for gel identification and sample orientation. Stain the gel with 0.0002% (w/v) ethidium bromide solution for 5 min. Photograph the gel under UV to visualize the 5S rRNA and tRNA bands to verify equal loading of samples.

Transfer to Membrane

1. miRNA samples that have been separated by PAGE are transferred to a positively charged nylon membrane (e.g., Hybond N+, Amersham Biosciences) electrophoretically (e.g., Semi Dry Transfer Cell System, Bio-Rad).
2. After photographing the gel, rinse the gel with water several times to remove ethidium bromide and then rinse once with 0.5× TBE buffer.
3. Cut two extra thick blotting papers (Bio-Rad, Hercules, CA) and a positively charged membrane to the size of the gel and equilibrate them in 0.5× TBE buffer for 30 min.
4. Prepare the transfer apparatus and moisten the surface of the electrodes (anode and cathode) with 0.5× TBE buffer.
5. On the anode (bottom), place a wet blotting paper, a membrane, and the gel and another blotting paper (described from the bottom to the top in order) (see Note 7).
6. Place the cathode on top of the assembled blotter-membrane-gel-blotter and close the apparatus (see Note 8).
7. Run the transfer apparatus at constant current (0.8 mA/cm^2 membrane dimension) for 60 min.
8. Turn off the apparatus and cut one corner of the membrane to identify sample orientation. Carefully remove the membrane and UV cross-link the miRNA. Dry membrane and use it for hybridization immediately or store between filter papers at room temperature until use.

*The protocol for gel electrophoresis of seed low-molecular-weight (LMW) RNA is originally from Dr. James Carrington's laboratory, Center for Genome Research and Biocomputing, Oregon State University.

3.2.3. Detection of miRNA (Northern Analysis)

Probe Synthesis

A description of miRNA probe synthesis using the mirVana™ miRNA Probe Construction Kit (Ambion) with modifications for DIG-labeled probes is presented.

1. DNA oligos for miRNA probe synthesis
   - Obtain the mature miRNA sequence from the miRNA database (http://asrp.cgrb.oregonstate.edu).
   - Add T_7 promoter (5′-cctgtctc-3′) at the 3′ end of mature miRNA sequence. To produce high-efficiency "AAA plus" miRNA probes, add AAA at both (5′ and 3′) ends of the miRNA sequence before adding the T_7 promoter sequence at the 3′ end.
2. Dissolve DNA oligomers for miRNA probe synthesis in water to a final concentration of 100 μM.
3. To make a double-stranded DNA template containing the T_7 promoter to be used for miRNA probe synthesis, mix 2 μL of DNA oligonucleotide, 2 μL of T_7 promoter primer (provided with the kit), and 6 μL of DNA hybridization buffer (provided with the kit) and heat the mixture at 70°C for 5 min.
4. Incubate the tube at room temperature for 5 min to allow annealing of the DNA oligomer and T_7 promoter primer.
5. To fill in and make a double-stranded DNA, add 2 μL of 10× Klenow reaction buffer (provided with the kit), 2 μL of 10× dNTP mix (provided with the kit), and 4 μL of water. Mix gently and then add 2 μL of Exo-Klenow DNA polymerase (provided with the kit) to the tube and mix by gently vortexing or pipetting.
6. Incubate the tube at 37°C for 30 min. This sample can be stored at –20°C until use.
7. For miRNA probe synthesis, mix 13 μL water, 1 μL double-stranded DNA template, 2 μL 10× Transcription buffer (provided with the kit), 2 μL T_7 RNA polymerase (provided with the kit), and 2 μL DIG RNA-labeling mix (Roche Applied Science) and incubate at 37°C for 60 min.
8. Add 1 μL RNase-free DNase (provided with the kit) to digest the DNA templates present in the miRNA probe synthesis mixtures and incubate at 37°C for 30 min.
9. Store DIG-labeled miRNA probes at –80°C until use.

Hybridization, Washing, and Detection

1. Prehybridize miRNA membranes (from the Subheading "Transfer to Membrane") in PerfectHyb ™Plus Hybridization Buffer (Sigma) at 42°C for 60 min (see Note 9).
2. Add 5 μL of miRNA probe to 2 mL hybridization buffer, boil, cool down to 42°C, and incubate at 42°C.
3. Cut three sides of a 0.11-mm-thick hybridization plastic bag (Kapak) and place the presoaked membrane (step 1) with the miRNA side up. Add miRNA probe in hybridization buffer (2 mL) on the membrane and seal the sides of bag carefully without trapping any air bubbles.
4. Hybridize at 42°C overnight.
5. Remove membrane from the hybridization bag and wash with 2× SSC, 0.2% (w/v) SDS, at 65°C three times for 20 min each.
6. Rinse the membrane with 1× maleic acid buffer containing 0.3% (v/v) Tween-20 at room temperature and then block the membrane with the same buffer containing 5% (w/v) nonfat milk at 25°C for 30 min.
7. Add anti-DIG antibody (1:15,000) to the blocking buffer and incubate at 25°C for 1 h.
8. Wash the membrane with 1× maleic acid buffer containing 0.3% (w/v) Tween-20 three times for 20 min.
9. Incubate the membrane for 5 min at room temperature in equilibration buffer to adjust environment (pH 9.5) for alkaline phosphatase reaction.
10. Place the membrane with RNA side facing up on a plastic sheet.
11. Apply chemiluminescent substrate for alkaline phosphatase (~10 μL cm^{-2} of membrane) and incubate at room temperature for 5 min.
12. Cover the membrane with plastic sheet (see Note 10).
13. Expose to X-ray film for >15 min (CDPstar) or >30 min (LumiPhos).

3.3. Expression Analysis of miRNA Target Genes in Seeds

3.3.1. Extraction of mRNA

1. Follow steps 1–6 of miRNA extraction procedure (Subheading 3.2.1).
2. Precipitate mRNA by keeping the LiCl-containing RNA sample at –20°C overnight.
3. Completely thaw sample and invert tube to mix well.
4. Centrifuge at 10,000×*g* for 2 min. Discard supernatant (see Note 11).
5. Add 1 mL of 80% (v/v) ethanol to wash the pellet. In many occasions, the pellet is not easily dissolved due to the presence

of coprecipitated PVP. In that case, physically break the pellet with a pipette tip and then vortex the sample to dissolve the pellet thoroughly.

6. Centrifuge the tube at 10,000 × *g* for 2 min and discard the supernatant (see Note 12).
7. Dry and resuspend the pellet in 10–20 μL of water and quantify RNA.
8. The mRNA sample may be used immediately or stored at –80°C.

3.3.2. Expression Analysis of mRNA

Once genes associated with seed dormancy or germination have been identified through microarray data mining, ordinary semi-quantitative real-time PCR or RT-PCR methods can be used to examine their expression levels at various stages of seed imbibition to validate their association with dormancy or germination.

3.4. Functional Analysis Using miRNA-Resistant Mutants

An efficient approach for characterizing miRNA-targeted genes is to facilitate "deregulation" of target genes by miRNA. This allows generation and overaccumulation of transcripts associated with miRNA-resistant genes. By characterizing phenotypes resulting from miRNA deregulation, the biological function of target genes and the involvement of miRNA in their regulation can be analyzed. Here, methods for introducing mutations into target gene sequences complementary to miRNA sequences are described.

3.4.1. Isolation of Genomic Fragments

1. Genomic sequences of genes of interest can be obtained from databases, such as http://www.arabidopsis.org and http://www.ncbi.nlm.nih.gov.
2. Design forward and reverse gene-specific primers to amplify miRNA target genes of interest, including 5′ upstream putative regulatory sequences (1–3 kb) and 3′ untranslated regions (see Notes 13 and 14).
3. Prepare *Arabidopsis* gDNA.
4. Amplify miRNA target genes of interest by PCR using gene-specific primers and gDNA (100 ng).
5. Clone the PCR products into cloning vectors.
6. Check for the accuracy of the cloned sequences.

3.4.2. Creation of Mutations

1. Identify the position of the miRNA target site within the sequence of the miRNA target gene of interest using databases (e.g., http://asrp.cgrb.oregonstate.edu).
2. Align the mature miRNA sequences from all loci to the complementary sequence of the miRNA target gene and identify the base pairs to be mutated at the 3′ end of the miRNA complementary site in the target gene (Fig. 1a). Mutations should be introduced in such a way that the miRNA mismatches

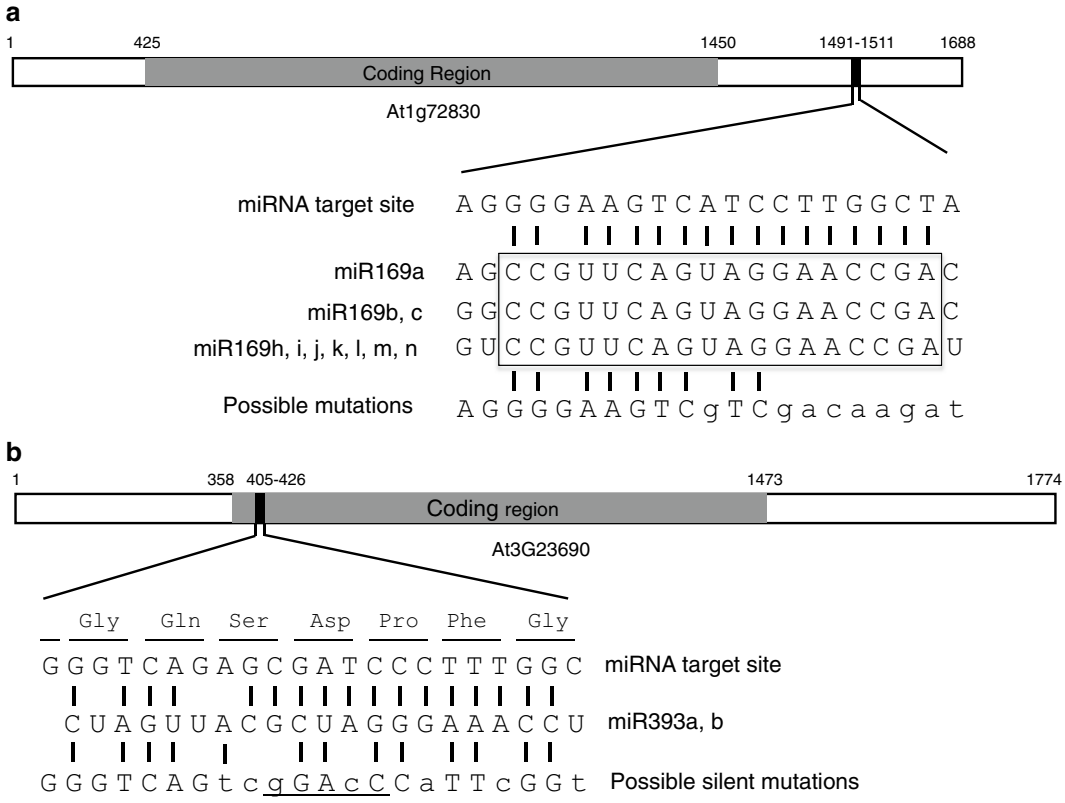
a
1
425
1450
1491-1511
1688
Coding Region
At1g72830
miRNA target site
A G G G G A A G T C A T C C T T G G C T A
miR169a
A G C C G U U C A G U A G G A A C C G A C
miR169b, c
G G C C G U U C A G U A G G A A C C G A C
miR169h, i, j, k, l, m, n
G U C C G U U C A G U A G G A A C C G A U
Possible mutations
A G G G G A A G T C g T C g a c a a g a t
b
1
358
405-426
1473
1774
Coding region
At3G23690
Gly Gln Ser Asp Pro Phe Gly
G G G T C A G A G C G A T C C C T T T G G C
miRNA target site
C U A G U U A C G C U A G G G A A A C C U
miR393a, b
G G G T C A G t c g G A c C C a T T c G G t
Possible silent mutations

Fig. 1. Introduction of mutations in the miRNA complementary sequences in target genes. (**a**) The miR169 complementary site of a target gene (At1g72830). Alignment of multiple miR169 sequences is shown with the conserved sequences highlighted in the box. Possible mutations in the 3′ end of the target gene (5′ end of miR169) are shown in lower-case letters. (**b**) The miR393 complementary site of a target gene (At3g23690). A *Sau*961 restriction site (underlined; gGAcC) is included (see text for detailed explanation about restriction site).

are sufficient to prevent hybridization. Mutations should be created without changing the amino acid sequences of the encoded protein if the miRNA target is within the coding sequence (Fig. 1b).

3. Amplify the miRNA target gene as two fragments with an overlap containing mutations at the miRNA complementary site (Fig. 2a). A forward primer designed to the 5′ end of the target gene (Primer F1) should be used with a reverse primer designed to create mutations in the miRNA complementary site (Primer R1). A forward primer containing mutations at the miRNA complementary site (Primer F2) should be used with a reverse primer designed to the 3′ end of the target gene (Primer R2). For detailed primer design, see Fig. 3. Note that R1 and F2 primers have complementary regions (underlined) that can be used to anneal the two fragments containing mutations

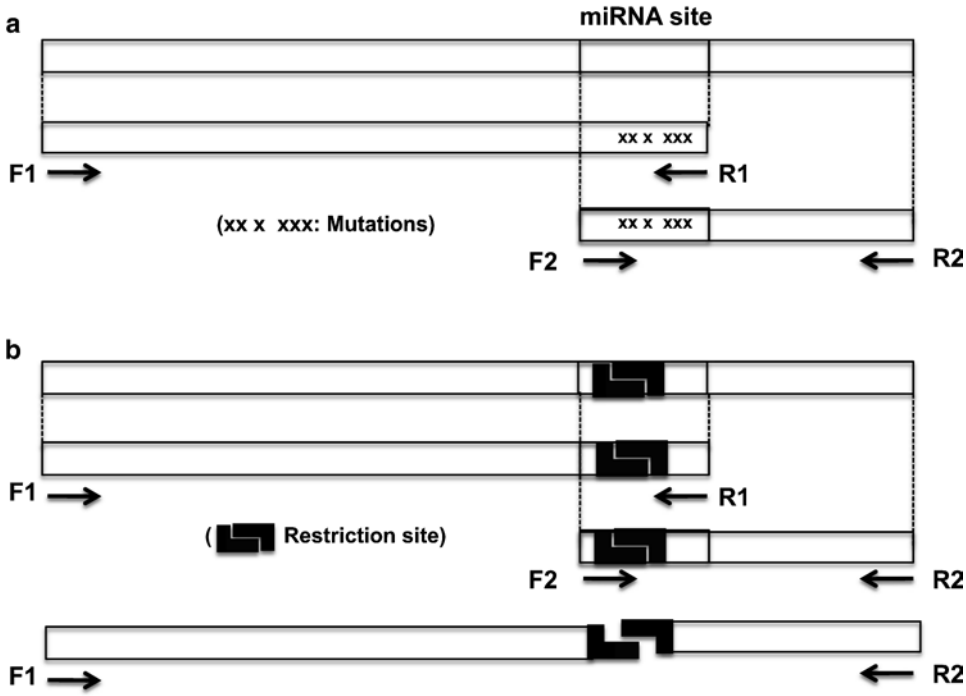

Fig. 2. Strategies to introduce mutations in miRNA target genes. (**a**) Introduction of mutations by two-step PCR. An miRNA target gene is amplified as two fragments with an overlap at the miRNA complementary site with mutations (xx x xxx). A forward primer designed to the 5′ end of the target gene (F1) is used with a reverse primer designed to create mutations in the miRNA complementary site (R1). A forward primer containing mutations at the miRNA complementary site (F2) is used with a reverse primer designed to the 3′ end of the target gene (R2). (**b**) Amplification of the full-length target gene containing mutations by restriction digestion and ligation. The same restriction site can be designed in the R1 and F2 primers and used to digest the F1/R1 and F2/R2 fragments amplified by PCR. The two purified digested products can be ligated and the final full-length fragment containing the desired mutation is amplified by PCR using F1 and R2 primers. For primer sequence design, see Fig. 3.

for site-directed mutagenesis (19). This can then be used with the outside primers to amplify the final full-length mutagenized fragment. A restriction site ligation-based method, instead of site-overlapping PCR, is described below.

4. When creating mutations, a restriction site that is not present in the original gene is introduced within the primer sequence. In Fig. 3, a *SalI* site is included in the R1 and F2 primers (see Note 15).
5. Amplify F1/R1 and F2/R2 fragments and PCR-purify.
6. Digest the two fragments separately with the restriction enzyme that recognizes the sequence introduced into the primers (Fig. 2).
7. Purify the two fragments and ligate them (use 100–500 ng each) with T_4 DNA ligase (Fig. 2).

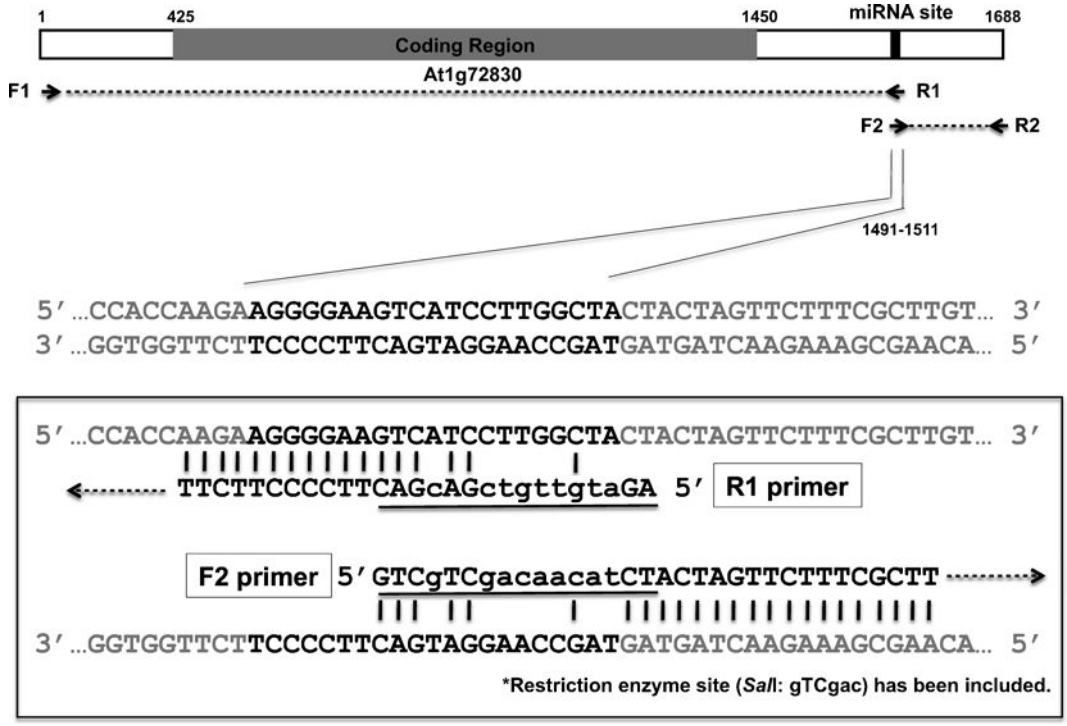

Fig. 3. Primer design to introduce mutations at the miRNA complementary site of the miRNA target gene. A reverse primer containing mutations (R1) is designed at the 3′ region of miRNA complementary sequence in the target gene and used with a forward primer designed to the 5′ end of the target gene (F1). A forward primer (F2) containing mutations and the sequence complementary to the R1 primer (underlined) is used with a reverse primer designed to the 3′ end of the target gene (R2). A *Sal*I site (gTCgac) is included in the R1 and F2 primers to facilitate digestion and allow subsequent ligation of the F1/R1 and F2/R2 PCR products to generate a template for amplifying the F1/R2 full-length fragment with mutations (see Fig. 2b).

8. Dilute the ligation mixture 250-fold and use as a template along with F1/R2 primers for PCR to amplify the final fragment containing the mutations (Fig. 2).
9. Clone the mutated gene into a cloning vector and sequence to check for errors.
10. Construct transformation vector and use to transform plants for phenotypic analysis.

4. Notes

1. Frozen seeds should be homogenized in the phenol-containing buffer A immediately. Allowing the tissues to thaw could cause RNA degradation.
2. After adding 1/3 volume of 8 M LiCl, the final concentration of LiCl is 2 M.

3. The supernatant after the 2 M LiCl precipitation contains LMW RNA, miRNA, and gDNA.
4. Concentrations of isopropanol necessary to separate LMW RNA from high-molecular-weight (HMW) RNA and genomic DNA may vary depending on plant species and tissues. A 40–50% isopropanol fraction usually gives good recovery of LMW RNA with minimum contamination of HMW RNA and gDNA.
5. The volumes of solutions for the 17% (w/v) acrylamide gel preparation are described for a mini gel (~6×10 cm). Adjust volumes depending on the size of your gel.
6. A 1.5-mm-thick spacer works better than a 1.0-mm-thick spacer because it provides wider wells, thereby reducing the height of the samples in the wells and minimizing diffusion of bands during electrophoresis.
7. Do not reposition the gel once it is in contact with the membrane. Repositioning the gel could cause fuzzy transfer images.
8. Air bubbles trapped between the gel and membrane can prevent transfer of RNA to the membrane. They can be removed by rolling a Pasteur pipette on top of the upper blotter paper from one side to the other.
9. Pre-boil the hybridization buffer and cool down to 42°C before soaking the membrane.
10. When CDPstar is used as a chemiluminescent substrate, gently squeeze out excess substrate solution. This reduces background on the X-ray film.
11. The supernatant from the LiCl precipitation sample kept at –20°C overnight during the mRNA extraction can be used for miRNA (LMW RNA) extraction. However, the LiCl pellet (by-product) from miRNA extraction that is frozen only for 10 min at –80°C is not suitable for isolating mRNA (HMW RNA). If both HMW and LMW RNA fractions are to be extracted from a single sample, LiCl precipitation must be done overnight.
12. The RNA pellet from *Arabidopsis* seeds may appear light brownish, but it is still suitable for Northern blot analysis and RT-PCR.
13. It is essential to use the native promoter for miRNA deregulation mutants.
14. Since the miRNA complementary sequences of some miRNA target genes occur outside their coding regions, it is important to include the 5′ and 3′ UTR in these constructs.
15. This restriction site is convenient to distinguish mutated (cleavable by the enzyme) from nonmutated (uncleavable) miRNA target genes in the expression analysis of transgenic plants.

Acknowledgments

We thank Dr. George Bassel for providing unpublished information on the bioinformatic analysis of seed dormancy- and germination-associated genes. This work was supported by a BOYSCAST grant (awarded to A.K.M.B.) and a National Science Foundation grant INB-0237562 (awarded to H.N.).

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Chapter 14

Proteomics and Posttranslational Proteomics of Seed Dormancy and Germination

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Abstract

The seed is the dispersal unit of plants and must survive the vagaries of the environment. It is the object of intense genetic and genomic studies because processes related to seed quality affect crop yield and the seed itself provides food for humans and animals. Presently, the general aim of postgenomics analyses is to understand the complex biochemical and molecular processes underlying seed quality, longevity, dormancy, and vigor. Due to advances in functional genomics, the recent past years have seen a tremendous progress in our understanding of several aspects of seed development and germination. Here, we describe the proteomics protocols (from protein extraction to mass spectrometry) that can be used to investigate several aspects of seed physiology, including germination and its hormonal regulation, dormancy release, and seed longevity. These techniques can be applied to the study of both model plants (such as *Arabidopsis*) and crops.

Key words: Proteomics, *Arabidopsis*, Seed, Dormancy, Germination, Longevity, Protein carbonylation

1. Introduction

The seed is the major form of dissemination in plants. It arises from the fertilized ovule and contains an embryo (the future plant) and various seed storage tissues (e.g., endosperm or megagametophyte, cotyledons, perisperm) that store reserve compounds necessary for the nutrition and growth of the embryo during and following germination. Various integuments, some of which form the seed coat, provide a shield to protect the embryo against biotic and abiotic stresses (1). For most species growing in temperate climates, a desiccation phase terminates seed development, after which the

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embryo enters a quiescent state permitting its storage and survival for many years (2). The seed, therefore, occupies a central position in the plant life cycle. The mature quiescent seed has a low moisture content (5–15%) and its metabolic activity is almost at a standstill. In the absence of dormancy (a physiological process that regulates germination), the dry quiescent seed commences germination upon encountering hydrated conditions that encourage metabolism, e.g., a suitable temperature and the presence of oxygen (3).

The completion of genome-sequencing projects, generation of large expressed sequence tag (EST) collections for several reference and crop plants, and the development of analytical methods for protein characterization have all led to the area of proteomics becoming a major field of functional genomics. Proteomic analyses of seed dormancy, longevity, and germination have been important for elucidating some of the gene products that may underlie seed quality and vigor in both reference plants and crops (1, 4). Indeed, the large amount of data demonstrated the power of descriptive biology to create novel functional insights, e.g., metabolic control and redox regulation of seed developmental processes, mechanisms accounting for desiccation tolerance or dormancy control (for reviews, see 1, 4). Concerning the germination process, proteomics proved to be the method of choice for establishing large-scale gene expression profiling since this process mainly relies on stored proteins and proteins that are synthesized de novo from the stored mRNAs (1, 5–8). Therefore, this developmental process is not primarily subject to transcriptional control, although some regulation may occur at this level, as in the modulation of germination rate and uniformity. It is anticipated that future proteomics research will address the question of specific tissue expression of seed proteins to understand better, in a systems biology approach, the functioning of a whole seed from the respective roles of its constituents, namely, the embryo, storage tissues, and seed coat (e.g., see 9). Another salient feature put forward by the proteomics data is the general importance of protein modification in seed development and germination, as exemplified by the very large number of proteins that proved to be the specific targets of thioredoxins (10, 11) and of oxidation through carbonylation (6–8, 12). In particular, the proteomics studies unraveled a new role for protein carbonylation in seed development and germination that has no counterpart in microorganisms and animals (6–8, 12, 13). These studies also revealed a link between reactive oxygen species (ROS) leading to protein carbonylation and redox regulatory events catalyzed by thioredoxin in seeds. The results suggested that a tuning of such protein modifications might exert a dramatic control upon seed development and germination, which will be the subject of future research.

Spectacular improvements in mass spectrometry have allowed the identification of proteins by de novo sequencing without prior knowledge of the corresponding gene sequences (14, 15).

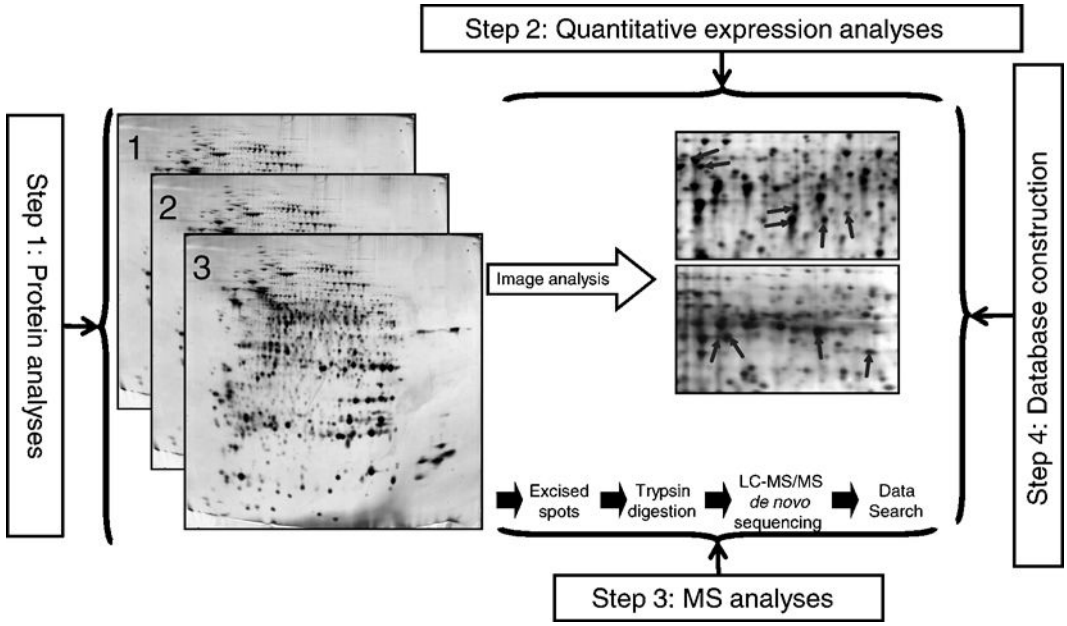

Fig. 1. Schematic presentation of the proteomics workflow. Protein samples made at least in triplicate and for a minimum of three independent extractions are processed by 2D-GE, followed by image and statistical analyses for detection of spots of interest, mass spectrometry, and database searching to identify the proteins. The final step is the database construction, allowing to establish specific protein expression signatures.

This offers the possibility of establishing large-scale protein profiles even in species for which genomic information is not yet available (e.g., see 9).

Here, we describe the proteomics protocols (such as protein extraction, 2D gels, and mass spectrometry, see Fig. 1) that can be used to investigate several aspects of seed physiology (5–9, 12, 13, 16–20), including germination and its hormonal regulation, dormancy release, or seed longevity, and their use is relevant for the study of both model plants (*Arabidopsis*) and crops (e.g., sunflower, sugar beet). These analyses are mainly based on quantitative changes in the accumulation of specific proteins as revealed by specific protein-staining reagents (e.g., silver nitrate or Coomassie blue). A dynamic proteomics approach that is based upon providing pulses of [^{35}S]-Met during the seed imbibition process is also described. This is a protocol that we have direct experience in and it offers the possibility to specifically characterize the de novo synthesized proteome during seed imbibition. From a methodological point of view, dynamic proteomics is much more potent than classical proteomics to reveal differential accumulation of proteins during seed imbibition (5–8).

2. Materials

2.1. Seed Production for Dormancy Analyses

1. A regulated growth chamber: Light (16-h fluorescent light period (NL-T8 58W/865/G13 [Radium, Germany], 250 µmol/m^2/s^1) and 8 h of darkness); temperature (21°C during light period and 19°C during dark period); humidity (50–60% RH).
2. Plant substrate (Tref Substrates with N/P/K=17/10/14, pH 6.2, The Netherlands).
3. Phytosanitary (PS) solution: 5 mL/L fungicide Previcur N (Bayer CropScience, France) and 400 mg/L insecticide Hortigard (Syngenta Agro, France) solubilized in water.

2.2. Preparation of Seed Samples

1. A laminar flow hood (ADS Laminaire, France).
2. Sterilization solution 1 (SS1): One tablet of Bayrochlor (Bayrol, Germany) dissolved in 40 mL of water.
3. Sterilization solution 2 (SS2): 5 mL of SS1 in 45 mL of 95% ethanol (Carlo Erba Reactifs, France).
4. One hundred percent ethanol (Carlo Erba Reactifs, France).
5. One hundred and fifty-mL coprology boxes (CML, France).

2.3. Seed Dormancy Release

1. Dormant seeds used are derived from *Arabidopsis thaliana* (L.) Heynh. ecotype Cape Verde Islands (Cvi). Seeds used in each experiment should originate from the same seed lot.
2. 10 mM KNO_3: 10 mg of KNO_3 (Sigma-Aldrich, France) dissolved in 10 mL of warm sterile water and filtered with 0.2-µm Minisart filter (Fisher Bioblock Scientific, France).
3. 10 mM KNO_2: 8.5 mg of KNO_2 (Sigma-Aldrich, France) dissolved in 10 mL of warm sterile water and filtered with 0.2-µm Minisart filter.
4. 10 mM 1-methyl-3-phenyl-5-(3-trifluoromethyl-(phenyl))-4-(1H)-pyridinone (fluridone): 32.9 mg of fluridone (Kalys, France) solubilized in 100 µL of dimethyl sulfoxide (DMSO; Kalys, France) and then diluted in 10 mL of sterile water (see Note 1).

2.4. Seed Aging

1. Transparent airtight tube carriers (Fisher Bioblock Scientific, France).
2. KCl (Sigma-Aldrich, France) saturated solution: 100 g of KCl salt solubilized in 200 mL of water and filtered to remove insoluble salt.
3. $CaCl_2$ (Sigma-Aldrich, France) saturated solution: 100 g of $CaCl_2$ salt solubilized in 200 mL of water and filtered to remove insoluble salt.
4. Oven regulated at 40°C (Jouan, France).

5. Fridges regulated at 15 and 20°C (Liebherr, France).
6. Testo 175-H2 temperature and humidity controller (Testo, France).

2.5. Seed Germination

2.5.1. Seed Germination on Agar Medium

1. Seed germination medium (GM): 7 g/L agar HP 696 (Kalys, France) and 580 mg/L MES (Sigma-Aldrich), pH 5.7, solubilized in water.
2. Parafilm (American National Can, Neenah, WI).
3. Petri dishes (Ø 55 mm; CML, France).
4. A controlled culture room with a 16-h photoperiod (NL-T8 58W/865/G13 [Radium, Germany], 250 µmol/m^2/s^1) regulated under standard conditions at 25°C (light period)/20°C (dark period) and a constant 70% RH.

2.5.2. Seed Germination on Paper

1. Absorbent paper (Roundfilter paper circles, Ø 45 mm; Schleicher & Schuell, Dassel, Germany).
2. Black membrane filters with a white grid (ME 25/31, Ø 45 mm; Schleicher & Schuell, Dassel, Germany).
3. A phytotronic chamber (Sanyo, France) with a 16-h photoperiod at 25°C (light period)/20°C (dark period) and a constant 70% RH (standard conditions).
4. Binocular microscope (Leica, France).
5. Petri dishes (Ø 55 mm; CML, France).
6. Parafilm (American National Can, Neenah, WI).

2.6. Protein Extraction

1. Porcelain mortar (Ø 75 mm) and pestle (Ø 23 mm, 84 mm length).
2. Ice and liquid nitrogen.
3. Extraction buffer: 18 mM Tris–HCl (Sigma-Aldrich), 14 mM Tris base (Sigma-Aldrich), 7 M urea (GE Healthcare), 2 M thiourea (Merck), 4% (w/v) 3-[3-cholamidopropyl-dimethylammonio]-1-propane-sulfonate (CHAPS, GE Healthcare), 0.2% (v/v) Triton X-100, and 1% (v/v) pharmalyte pH 3–10 carrier ampholytes (GE Healthcare). This solution should be stored in 10-mL aliquots at –20°C.
4. Protease inhibitor cocktail Complete Mini (Roche Diagnostics) and 14 mM dithiothreitol (DTT; GE Healthcare), stored at –20°C.
5. DNase I (53 units/mL; Roche Diagnostics) and RNase A (4.9 Kunitz units/mL; Sigma-Aldrich), stored at –20°C.

2.7. Protein Separation

2.7.1. One-Dimensional SDS-Polyacrylamide Gel Electrophoresis

1. Separating buffer (10×): 3.75 M Tris–HCl, pH 8.8, 1% (w/v) sodium dodecyl sulfate (SDS), stored at room temperature.
2. Stacking buffer (10×): 1.25 M Tris–HCl, pH 6.8, 1% (w/v) SDS, stored at room temperature.

3. Thirty percent (v/v) acrylamide/bisacrylamide solution (Bio-Rad), 10% (w/v) ammonium persulfate (GE Healthcare), *N,N,N,N*′-tetramethyl-ethylenediamine (TEMED, Bio-Rad), isopropyl alcohol (Carbo Erba), and prestained SDS-polyacrylamide gel electrophoresis (PAGE) standard molecular weight markers (Bio-Rad).
4. Running buffer (10×): 250 mM Tris base, 920 mM glycine for electrophoresis, both ≥99% pure (Sigma-Aldrich), 1% (w/v) SDS (GE Healthcare), stored at room temperature.

2.7.2. Two-Dimensional Gel Electrophoresis

1. Immobiline DryStrips pH 3–10 NL, 18 cm (GE Healthcare), stored at –20°C.

Isoelectric Focusing

2. Rehydration buffer (see Note 2): 18 mM Tris–HCl, 14 mM Tris base, 7 M urea (GE Healthcare), 2 M thiourea (Merck), 4% (w/v) CHAPS (GE Healthcare), 20 mM DTT (GE Healthcare), 2% (v/v) Triton X-100, and 1% (v/v) pharmalyte pH 3–10 carrier ampholytes (GE Healthcare).
3. Mineral oil (Sigma-Aldrich), stored at room temperature.

Equilibration

1. Reducing buffer: 6 M urea, 30% (v/v) glycerol, 50 mM DTT, 2.5% (w/v) SDS, 0.15 M bis(2-hydroxyethyl) iminotris (hydroxymethyl) methane (BisTris, Sigma-Aldrich), and 0.1 M HCl, pH 8.8.
2. Washing buffer: 6 M urea, 30% (v/v) glycerol, 2.5% (w/v) SDS, 0.15 M BisTris, and 0.1 M HCl, pH 8.8.
3. Blocking buffer: 6 M urea, 30% (v/v) glycerol, 4% (w/v) iodoacetamide, 2.5% (w/v) SDS, 0.15 M BisTris, and 0.1 M HCl, pH 8.8.

SDS-PAGE

1. Denaturing agarose: 1% (w/v) low-melting agarose (Gibco BRL), 0.4% (w/v) SDS, 0.15 M BisTris, and 0.1 M HCl.
2. Polyacrylamide gels: 10% (v/v) acrylamide, 0.33% (w/v) piperazine diacrylamide, 0.18 M Tris base, 0.166 M HCl, 0.07% (w/v) ammonium persulfate, and 0.035% (v/v) TEMED.
3. Running buffer: 25 mM Tris base, 200 mM taurine (Sigma-Aldrich), and 0.1% (w/v) SDS, pH 8.3.

2.8. Protein Staining

1. Fixing buffer: 30% (v/v) ethanol and 10% (v/v) acetic acid.
2. Sensitizer solution: 0.02% (w/v) sodium thiosulfate.
3. Silver staining solution: 0.12% (v/v) silver nitrate and 0.0003% (v/v) formaldehyde.
4. Development solution: 3% (w/v) potassium carbonate and 0.0003% (v/v) formaldehyde.
5. Stop solution: 30% (w/v) EDTA.

6. Preserving solution: 30% (v/v) ethanol and 5% (v/v) glycerol.
7. GelCode blue stain reagent (Pierce).

2.9. Western Blotting for Detection of Carbonylated Proteins

1. Whatman paper (7×9.5 cm) and nitrocellulose membrane (Bio-Rad).
2. TBS (1×): 50 mM Tris–HCl and 150 mM NaCl, pH 7.5.
3. TBS-Tween (1×): 50 mM Tris–HCl, 150 mM NaCl, and 0.01% (v/v) Tween 20, pH 7.5.
4. OxyBlot™ Oxidized Protein Detection Kit (CHEMICON International, USA): Kit components: 10× 2,4-dinitrophenylhydrazine (DNPH); neutralization solution; mixture of standard proteins with attached DNP residues; primary antibodies: rabbit anti-DNP antibodies; secondary antibodies: horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgGs; 10× derivatization-control solution.
5. Hyperfilm ECL 18×24 cm and ECL™ Western Blotting Detection Reagents (GE Healthcare).

2.10. Protein Radiolabeling

1. Safety equipment: Plexiglas screens, Benchcoat.
2. [^{35}S]-Met (1.85 MBq; ICN Biomedicals, SARL).
3. Sheet of Saran wrap (VWR international SAS).
4. Sheet of cellophane model Gel Dryer (Bio-Rad).
5. Low energy storage phosphor screen and exposure cassette (Kodak).

2.11. Protein Identification by Mass Spectrometry

All solutions should be prepared with high-quality chemicals as well as ultrapure deionized water for both sample preparation and mass spectrometry (MS) analysis to identify proteins in the low femtomole range.

2.11.1. Sample Preparation: In-Gel Digestion of Proteins (21)

1. Destaining solution for silver-stained gels: 1:1 mixture of 30 mM $K_3Fe(CN)_6$/100 mM $Na_2S_2O_3$ sodium thiosulfate solutions. Solutions can be stored in glass (ambered for potassium ferricyanide) or plastic bottles for several months. The 1:1 mixture must be made freshly before destaining.
2. Reduction/alkylation and trypsin hydrolysis: Acetonitrile (ACN), formic acid, 50 mM ammonium bicarbonate, 10 mM DTT, 55 mM iodoacetamide, sequencing-grade trypsin (12.5 ng/µL for Coomassie blue-stained gels or 6.25 ng/µL for silver-stained gels).
3. Equipment: Oven regulated at 56 and 37°C, vortex, centrifuge, SpeedVac concentrator.

2.11.2 MALDI-TOF (MS)

1. Reagents for target preparation (Bruker Daltonics, Bremen, Germany): Peptide standard mixture (angiotensin II, angiotensin I, substance P, bombesin, adrenocorticotropic hormone

(ACTH) clip 1–17, ACTH clip 18–39, somatostatin 28); covered mass range: ~1,000–3,200 Da.

2. Matrix: The most often used matrix for peptide analysis is α-cyano-4-hydroxycinnamic acid (ACHCA) in 70/30 ACN/0.1% (v/v) trifluoroacetic acid (TFA) at 5 mg/mL.
3. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer.

2.11.3. Tandem Mass Spectrometry

1. Chromatographic columns: Analytical (Atlantis dC18, 3 μm, 100 Å, 75 μm × 150 mm Nano Ease, Waters, MA) and preconcentration (Atlantis dC18 trap column 5 μm, 0.18 × 23.5 mm).
2. Chromatographic elutants for HPLC separation: A = 2% ACN/98% H_2O containing 0.1% (v/v) formic acid and B = 80% ACN/20% H_2O containing 0.1% formic acid. C = 0.1% formic acid used for desalting on the preconcentration column.
3. CapLC nano-HPLC system coupled to a Q-TOF mass spectrometer equipped with a Z-spray electrospray source (Waters, MA).

3. Methods

3.1. Seed Production for Dormancy Analyses

1. Sterilized seeds are shown on Petri dishes containing germination medium sealed with Parafilm and stratified for 4 days at 4°C.
2. Petri dishes are transferred into a controlled culture room and maintained under germination conditions for 2 weeks.
3. Two-week-old seedlings are individually transplanted onto substrate pretreated with PS solution (1 L) for seed production.
4. Plants are watered three times a week throughout a 60-day period to obtain full maturation of seeds on mother plants.
5. Watering is stopped for 3 weeks to allow complete seed maturation and desiccation before harvest. Seeds are stored in 15-mL tubes at 4°C before protein sample preparation.

3.2. Preparation of Seed Samples

1. Mature seeds are harvested after full maturation from dehydrated siliques.
2. The collected seeds are systematically sterilized under a laminar flow hood. Aliquots of seed samples are placed into 1.5-mL tubes (approximately 10 mg per tube) containing 1 mL of SS2 and stored for 10 min at room temperature with occasional shaking. The solution is discarded and the seeds are washed twice with 1 mL of 100% ethanol. Then, the solution is discarded

and the tubes are stored open at room temperature for 8 h until complete drying of seeds occurs (see Note 3).

3. The sterilized seeds are either used immediately in the dried state or stored in plastic coprology boxes at 7°C, 40% RH, in a storage cabinet (see Note 4).

3.2.1. Seed Dormancy Release

Dormancy of freshly harvested Cvi seeds is characterized by a low germination percentage under standard germination conditions. This primary dormancy can be released in seeds maintained in the air-dry state by after-ripening or by hydration of the seeds either in water at cool conditions (moist chilling or "stratification") or in the presence of various chemicals.

After-Ripening

1. Dormant Cvi seeds are sampled into 1.5-mL tubes.
2. Nondormant (ND) seeds are obtained by treatment of seeds for seven months at 36% RH (20°C) in airtight tube carriers containing a $CaCl_2$ saturated solution.
3. Dormancy release is checked by a germination assay as described in Subheading 3.2.3 "Seed germination on Agar (For Studies with Dormant Seeds)."
4. Resulting after-ripened seeds can be stored at 4°C at least for 1 year before use.

Moist Chilling ("Stratification")

1. Dormant Cvi seeds are sown in 55-mm Petri dishes containing 7 mL of GM.
2. Petri dishes are sealed with Parafilm, and complete dormancy release is obtained by incubating Petri dishes for 4 days in the dark at 4°C.
3. Seeds in Petri dishes are immediately transferred to a controlled culture room maintained at germination conditions as described in Subheading "Seed Germination on Agar (For Studies with Dormant Seeds)."

Chemical Treatments for Dormancy Breakage

1. GM supplemented with either 7 mM KNO_3, 7 mM KNO_2, or 10 μM fluridone [an abscisic acid (ABA) biosynthesis inhibitor] is prepared by dilution and homogenization of the 10 mM nitrate, 10 mM nitrite, or 10 mM fluridone stock solutions, respectively, in the GM maintained at 60°C prior to agar solidification.
2. Dormant Cvi seeds are sown in 55-mm Petri dishes containing 7 mL of the appropriate GM supplemented by nitrate, nitrite, or fluridone.
3. Petri dishes are sealed with Parafilm and incubated in a controlled culture room maintained at germination conditions as described in Subheading 3.2.3 "Seed germination on Agar (For Studies with Dormant Seeds)."

3.2.2. Seed Aging

Natural Aging

Freshly harvested *Arabidopsis* seeds (1 g) are stored in hermetic containers in the dark in a cold room at 4°C for periods extending to several years.

Artificial Aging: Controlled Deterioration Treatment

Seed vigor as assessed by artificial aging assays has been described by Delouche and Baskin (22) for estimating the storage potential of seed lots and is now accepted for all species with orthodox storage behavior. Later, aging tests were also shown to indicate the relative field emergence of seed lots of numerous species. The principle of these tests is to expose seed samples for a defined period of time to an unfavorable environment, e.g., of high temperature and high seed moisture content. After this aging period, high-vigor seeds are expected to still show high germination, whereas low-vigor seeds are expected to exhibit a considerable decrease in germination (23).

The controlled deterioration treatment, known as CDT, is presumed to mimic natural seed aging (24). The CDT is widely used as a vigor assay for numerous seed species and has been described for *Arabidopsis* seeds by Tesnier et al. (25). We have used this protocol with slight modifications to deteriorate in a controlled manner *Arabidopsis* seeds (8, 19, 26).

1. Aliquots of surface-sterilized seeds are placed into 1.5-mL tubes, and the tubes are put into a rack and allowed to equilibrate for 3 days at 84% RH (15°C) in airtight tube carriers containing a KCl saturated solution. Temperature and humidity are measured with a digital controller placed inside the tube carriers.
2. Day 0 controls are immediately dried back for 3 days at 36% RH (20°C) in airtight tube carriers containing a $CaCl_2$ saturated solution.
3. The storage treatment is performed for various times (from 1 to 12 days) by storing the seeds at 84% RH (40°C) in airtight tube carriers containing a KCl saturated solution.
4. After each storage time, seeds are dried back as the control and then stored at 4°C before running germination experiments.

3.2.3. Seed Germination

Seed Germination on Agar (For Studies with Dormant Seeds)

1. Mature dormant seeds are sown in 55-mm Petri dishes containing GM.
2. In standard conditions, Petri dishes are sealed with Parafilm and incubated in a controlled culture room under a 16-h photoperiod at 25°C (light period)/20°C (dark period) and a constant 70% RH.
3. Germination measurements are scored three to five times per day. Seeds are considered germinated as soon as endosperm rupture is observed using a binocular microscope (see Note 5).

Seed Germination on Paper (For Studies with Nondormant Seeds)

Seeds of the nondormant *A. thaliana* ecotype Landsberg *erecta* (L*er*) are used to characterize the seed germination proteome. Here, germination assays are carried out on three replicates of 64 seeds.

1. Seeds are incubated at 25°C under an 8-h photoperiod (see Note 6) on three sheets of absorbent paper and a black membrane filter with a white grid wetted with 1.3 mL of distilled water in covered Petri dishes.
2. Germination measurements are scored three to five times per day. Seeds are considered as germinated as soon as endosperm rupture is observed using a binocular microscope (27–31; see Note 5).

3.3. Protein Extraction

Total soluble protein extracts are prepared from *Arabidopsis* seeds exhibiting contrasting levels of dormancy or vigor or after various times postimbibition.

1. Seeds (100 mg) are ground using mortar and pestle in liquid nitrogen.
2. Total soluble proteins are extracted at 4°C in 640 µL of extraction buffer and 110 µL of protease inhibitor cocktail. The protein extract is transferred from the mortar to an Eppendorf tube (1.5 mL).
3. After 10 min at 4°C, DNase I, RNase A, and DTT are added, the protein extract is stirred for 1 h, and then centrifuged (35,000 × *g*, 10 min) at 4°C.
4. The supernatant is submitted to a second clarifying centrifugation as above.
5. The final supernatant corresponds to the total soluble protein extract. Protein concentrations in the various extracts are measured using the Bradford protein assay (32). Bovine serum albumin is used as a standard.

3.4. Protein Separation

3.4.1. One-Dimensional SDS-PAGE

PAGE separates molecules in complex mixtures according to size and charge. During electrophoresis, there is an intricate interaction of samples, gel matrix buffers, and electric current resulting in separate bands of individual molecules. These instructions assume the use of a Mini-PROTEAN® 3 Cell (Bio-Rad, Code No. 165–3302) connected to PowerPac™ HC power supply (Bio-Rad, Code No. 164–5052). Mini-PROTEAN® 3 components are not compatible with acetone, ethanol, or butanol. It is critical that the glass plates for the gels are scrubbed clean with a rinsable detergent after use and rinsed extensively with distilled water.

1. Prepare a 1-mm-thick 10% gel by mixing 3 mL of 10× separating buffer with 10 mL of the acrylamide/bisacrylamide solution, 17 mL water, 100 µL of the ammonium persulfate solution, and 20 µL TEMED.

2. Pour the gel, leaving space for a stacking gel, and overlay with 1 mL of isopropyl alcohol. The gel is polymerized in about 30 min.
3. Remove the isopropyl alcohol and rinse the top of the gel twice with water.
4. Prepare the stacking gel by mixing 1 mL of 10× stacking buffer with 1.3 mL of the acrylamide/bisacrylamide solution, 7.6 mL water, 50 µL ammonium persulfate solution, and 10 µL TEMED.
5. Use about 0.5 mL of the stacking gel solution to quickly rinse the top of the gel and then load the stacking gel and insert the comb. The stacking gel is polymerized in about 30 min.
6. Prepare the running buffer by diluting 40 mL of the 10× running buffer with 400 mL of Milli-Q water. Once the stacking gel is polymerized, carefully remove the comb.
7. Assemble all the components of the Mini-PROTEAN 3 Electrophoresis Module Assembly.
8. Add the running buffer to the upper and lower chambers of the gel unit and load 20 µL of samples in each well.
9. Include one well for 5 µL of the mixture of prestained molecular weight markers.
10. Complete the assembly of the gel unit and connect to the PowerPacTM HC power supply. Gels run for 30 min at 10 mA/gel through the stacking gel and 1.5 h at 25 mA/gel through the separating gel.

3.4.2. Two-Dimensional Gel Electrophoresis

Isoelectric Focusing

This protocol is suitable for first-dimension isoelectric focusing (IEF) of proteins on the Multiphor II Electrophoresis unit connected to an EPS 3501 XL power supply and to a MultiTemp™ III thermostatic circulator (all equipment available at GE Healthcare). The Multiphor II Electrophoresis unit is supplied with a ceramic cooling plate, strip electrodes, and an electrode holder. This system provides a flexible and economical alternative for performing first-dimension IEF.

1. Rehydration: Proteins are first separated by electrophoresis according to charge. Immobilized pH gradients (IPGs) for IEF were introduced in 1982 (33). IEF is conducted with samples containing approximately 150 mg protein (equivalent to a total protein extract derived from 100 seeds). Protein extracts are separated using IPG gel strips forming an immobilized nonlinear pH gradient from 3 to 10. Immobiline DryStrip gels are rehydrated individually in the Reswelling tray (GE Healthcare, Code No. 80-6371-84). The rehydration buffer (350 µL) containing 150 mg protein is placed in each tray channel. Carefully remove the cover foil from the Immobiline

DryStrip and place it in the tray channel with the gel side down. Avoid trapping of air bubbles under the strip. Overlay the strip with mineral oil. This rehydration step runs for 14 h at 20°C. Up to 12 strips are rehydrated simultaneously. Remove the mineral oil. The rehydrated IPG strips are immediately transferred to the Reswelling cassette (GE Healthcare). Place the strips with the pointed (acidic) end at the top of the tray near the red electrode (anode). The blunt end should be at the bottom of the tray near the black electrode (cathode). Place moistened Whatman gel blotting paper strips across the cathodic and anodic ends of the aligned IPG strips. The Whatman gel blotting papers strips must at least partially contact the gel surface of each IPG strip. Each electrode has a side marked red (anode) or black (cathode). Align each electrode over Whatman gel blotting paper strips, ensuring that the marked side corresponds to the side of the tray giving electrical contact. When the electrodes are correctly aligned, press them down to contact the Whatman gel blotting paper strips. Overlay IPG strips with mineral oil. Perform the IEF at 20°C for 1 h at 300 V and for 7 h at 3,500 V.

2. Equilibration: After removing mineral oil, position the Reswelling cassette on a rocker. Equilibrate the gel strips for 30 min in 100 mL of the reducing buffer, 5 min in 100 mL of the washing buffer, and 20 min in 100 mL of the blocking buffer (34, 35).

SDS-Polyacrylamide Gel Electrophoresis

SDS-PAGE represents the second-dimension separation of the 2D-PAGE. Large-scale proteome analysis usually requires simultaneous electrophoresis of batches of second-dimension SDS-PAGE gels to maximize the reproducibility of 2D electrophoresis protein profiles. This requirement is most easily met using multiple, vertical second-dimension SDS-PAGE systems. This protocol presents a method for preparing and running 12 vertical SDS gels using the Ettan™ DALTtwelve system (GE Healthcare). The Ettan DALTtwelve system is controlled by the power supply/control unit. The unit supplies a maximum power output of 200 W with a maximum of 600 V or 1 A. The temperature control range is 10–50°C. Equilibrated gel strips are placed on top of vertical polyacrylamide gels. Denaturing agarose is loaded onto the gel strips. After agarose solidification, electrophoresis is performed at 10°C in the running buffer for 1 h at 35 V and for 14 h at 100 V. Proteins are then separated according to size. For each condition analyzed, 2D gels are made at least in triplicate and from three independent protein extractions.

3.5. Protein Staining

3.5.1. Silver Staining

Silver staining is the most sensitive nonradioactive method to detect proteins; this procedure is amenable to the detection of proteins that are at concentrations below 1 ng. Silver staining is a

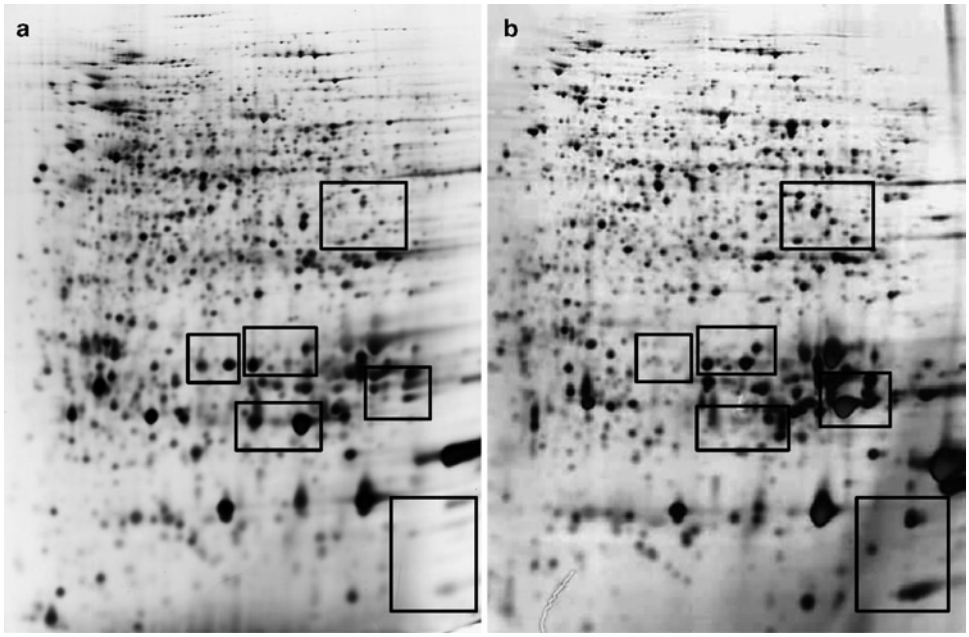

Fig. 2. Silver-stained 2D gels of total proteins from two accessions of *Arabidopsis* dry mature seeds (from 20): (**a**) Cap Verde Island dry mature seed proteome (dormant seeds), (**b**) Landsberg *erecta* dry mature seed proteome (nondormant seeds). Black frames indicate some examples of major protein variations, which could arise either from sequence polymorphism between the two accessions or from proteome differences between dormant and nondormant states of the seeds from the two accessions (for more details, see 20).

complex multistep process utilizing numerous reagents for which quality is critical (36). Therefore, use reagent-grade chemicals and prepare all solutions fresh. An illustration of silver-stained 2D gels is shown in Figs. 2 and 3. By omitting glutaraldehyde from the sensitizer and the diminution of formaldehyde content in the silver nitrate solution, the method becomes compatible with mass spectrometry analysis (21).

1. After SDS-PAGE, remove the gel from the cassette and place into a tray containing appropriate volume of fixing buffer. Soak the gel in this solution for 45 min. Fixation limits protein movement from the gel matrix and removes interfering ions and detergent from the gel.
2. Remove the fixing buffer and wash the gel in distilled water four times for 10 min.
3. Incubate the gel in the sensitizer solution for 1.5 min with gentle rotation. It increases the sensitivity and the contrast of the staining.
4. Remove the sensitizer solution and wash the gel twice, for 1 min each time, in distilled water.

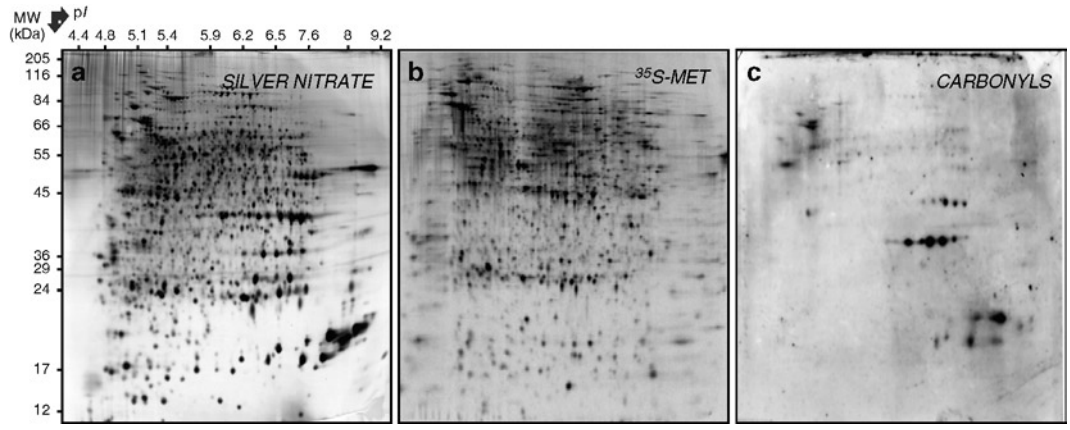

Fig. 3. Total soluble proteins revealed by silver staining (**a**), de novo protein synthesis pattern (**b**), and protein carbonyl pattern (**c**) during sugar beet seed germination. Note the huge differences in the three patterns showing that steady-state protein levels (**a**) are not equivalent to protein dynamic changes (**b**). Also panel (**c**) shows the specificity of protein carbonylation, meaning that not all seed proteins are subject to such a modification during germination. (J. Catusse, C. Job, D. Job, unpublished).

5. Remove the distilled water. Add the silver staining solution and agitate for 10 min to allow the silver ions to bind to proteins.
6. Remove the silver staining solution and wash the gel twice, for 1 min each time, in distilled water to remove the excess of unbound silver ions.
7. Reveal the protein spots by incubating the gel in the development solution and agitate for 10–20 min and wash the gel twice, for 1 min each time, in distilled water. The reaction can be stopped with the stop solution as soon as the desired intensity of the spots is reached.
8. Remove the distilled water. Add the stop solution directly to the gel. Gently agitate the gel during 45 min and wash the gel twice, for 10 min each time, in distilled water. Moist gels are placed in preserving solution at 4°C prior to drying.

3.5.2. Coomassie Blue Staining

Coomassie blue staining, although 50- to 100-fold less sensitive than silver staining, is a relatively simple method and is also more quantitative than silver staining. Hence, Coomassie blue is preferable when accurate relative amounts of proteins are to be determined by densitometry. Colloidal staining methods are recommended because they show the highest sensitivity, down to 100 ng per protein spot (37).

1. After SDS-PAGE, remove the gel from the cassette and place into a tray containing appropriate volume of fixing buffer. Soak the gel in this solution for 45 min.
2. Remove the fixing buffer and wash the gel in distilled water four times for 10 min.

3. Remove distilled water and add GelCode blue stain reagent for 1 h.
4. Remove the GelCode blue stain reagent and wash the gel in distilled water six times for 10 min. Moist gels are placed in preserving solution at 4°C prior to drying.

3.6. Western Blotting for Detection of Carbonylated Proteins

The OxyBlot™ Kit is ideal for the sensitive and rapid detection of proteins modified by oxygen-free radicals and other reactive species. As a consequence of this modification, carbonyl groups are introduced into protein side chains. By using this protocol, the carbonyl groups are derivatized with DNPH and then detected by western blotting with antibodies specific to the attached DNP moiety of the proteins. An illustration of seed carbonylated proteome is shown in Fig. 3.

1. In our experience, the most efficient method is to perform the DNP derivatization during 15 min directly into the IPG gel strips after the first-dimension IEF of proteins has been carried out.
2. Then, after the incubation with the DNPH reagent, the IPG gel strips are submitted to the equilibration step and the second dimension is performed followed by western blotting. These directions assume the use of the Multiphor II NovaBlot unit (GE Healthcare) connected to EPS 3501 XL power supply for the protein transfer from the acrylamide gel to the Hybond ECL nitrocellulose membrane (GE Healthcare).
3. The Hybond ECL nitrocellulose membrane is incubated in 50-mL blocking buffer for 1 h at RT on a rocking platform.
4. The blocking buffer is discarded and the membrane quickly rinsed prior to addition of a 1:5,000 dilution of the rabbit anti-DNP antibodies in TBS-Tween for 1 h at RT on a rocking platform.
5. The primary antibodies are then removed and the membrane washed three times for 5 min, each with 50 mL of TBS-Tween.
6. The HRP-conjugated goat anti-rabbit secondary antibodies (Sigma-Aldrich) are freshly prepared for each experiment as a 1:10,000-fold dilution in blocking buffer and added to the membrane for 30 min at room temperature on a rocking platform.
7. Then, the membrane is washed six times for 10 min each with TBS-Tween. During the final wash, 2-mL aliquots of each component of the ECL™ reagent (GE Healthcare) are warmed separately to room temperature and the remaining steps are done in a darkroom under safelight conditions.
8. Once the final wash is removed from the blot, the ECL™ reagents are mixed together and then immediately added to the blot, which is then rotated by hand for 1 min to ensure even coverage.

9. The blot is removed from the ECL™ reagents, blotted with Kim-Wipes, and then placed between the leaves of an acetate sheet protector that has been cut to the size of an X-ray film cassette. The acetate containing the membrane is then placed in an X-ray film cassette with Hyperfilm ECL for a suitable exposure time, typically for a few minutes.

3.7. Protein Radiolabeling (Dynamic Proteomics)

1. Proteins synthesized in vivo are radiolabeled by imbibing seeds on water for 1 day in the presence of [^{35}S]-Met (5–8).
2. Protein synthesis is globally quantified by TCA precipitation of aliquots of reaction mixtures spotted on Whatman GF/C filters.
3. After ten washing steps for 1 min in cold 5% (v/v) TCA and 0.04 M sodium pyrophosphate and two washing steps in absolute ethanol, filters are dried and counted for radioactivity in a liquid scintillation counter.
4. Radiolabeled protein extracts are prepared and submitted to 2D-gel electrophoresis (GE) as described above.
5. Proteins on the 2D gels are stained by silver nitrate (see above).
6. Then, stained 2D gels are dried for 1 week at room temperature in a sandwich composed of, from bottom to top, one sheet of cellophane model Gel Dryer, 2D gel, one sheet of Saran wrap, and one sheet of cellophane model Gel Dryer.
7. After drying, the upper sheet of cellophane and the Saran sheet are peeled and gels are submitted to PhosphorImager analysis (Molecular Dynamics Storm 840 PhosphorImager, Amersham Biosciences). Labeled 2D protein patterns are scanned as described above for the silver nitrate-stained gels. An illustration of neosynthesized proteome is shown in Fig. 3.

3.8. Protein Identification by Mass Spectrometry

3.8.1. Sample Preparation: In-Gel Digestion of Proteins

1. Slice the gel into small pieces of 1×1 mm and transfer to an Eppendorf tube. Take special care to avoid keratin contamination from hair, skin, or dust (work under a laminar flow cabinet, use nonpowdered gloves, and tie back hair if necessary).
2. Gels are stained and destained. For silver-stained gels, incubate gels or gel pieces in destaining solution with gentle shaking until no color is visible, approximately for 5–15 min. The gels become yellow. Wash the gels four to five times for 15 min with Milli-Q water until they become transparent and show no background color. For Coomassie blue-stained gels, wash the gel pieces sequentially with water (10 min), ACN (10 min), and 50 mM NH_4HCO_3 (10 min). Repeat the washes until the bulk of the Coomassie blue stain is removed, although it does not have to be completely removed at this stage.

3. Gels are reduced. Cover gel pieces with ACN and incubate for 10 min or until gel pieces become opaque. Remove ACN and rehydrate the gel with 50 µL DTT solution. Incubate at 56°C for 45 min.
4. Proteins in gels are alkylated. Rinse gels with 50 µL ACN and replace quickly with the iodoacetamide solution. Incubate in the dark at room temperature for 20 min. Remove the supernatant and cover the gel with ACN for complete dehydration of the gel.
5. Gel proteins are subjected to trypsin hydrolysis. Remove ACN and completely dry the gel in a Savant SpeedVac concentrator. Hydrate the gel with 4–5 µL of the trypsin solution and cover gel with 10–20 µL of 25 mM NH_4HCO_3, depending on the gel volume and incubate overnight at 37°C.
6. Peptides are recovered. Stop the hydrolysis by adding formic acid to a final concentration of 1% (v/v) and add an equal volume of ACN. Incubate for 15 min, then remove the supernatant, and dry in the SpeedVac concentrator. Dried peptides can be stored at –20°C for several weeks and reconstituted in 0.1% (v/v) formic acid or TFA for LC-MS/MS or MALDI analysis, respectively.

3.8.2. Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry

MALDI-TOF MS is used for protein identification by peptide mass fingerprinting (PMF). It is, thus, limited to the proteins of organisms for which sufficient database information is available.

1. MALDI spots are prepared with the conventional dry droplet method. Deposit 1 µL of the peptide digest solution on the target well. Add an equal volume of matrix solution and allow drying as a crystalline coating on the metal target at room temperature. For higher reproducibility, the target can also be dried under vacuum. Deposit the standard peptide mixture on the next well and load the target into the MALDI source.
2. MALDI analysis is conducted. Calibrate the MALDI-TOF instrument with the standard peptide mixture and apply this external calibration to the sample. The spectrum can also be internally calibrated afterward using tryptic autolysis of peptides and/or keratin peptides or any other contaminant of known mass.
3. Data is analyzed for PMF. Process the MALDI spectrum (background noise subtraction, smoothing, and deisotoping) to extract the monoisotopic peak list values. Match the peak list to the databank of your choice using classical databank search engines (such as MASCOT on matrixscience, MS-FIT on UCSF, and Aldente at Genebio). Critical search parameters are: monoisotopic values of [M+H]+ peaks, enzyme specificity (trypsin cleavage at C-terminal of Lys and Arg residues if not followed by a Pro residue), mass tolerance (should never exceed 100 ppm), modification (fixed alkylation of Cys to carbamidomethyl Cys and variable

oxidation of Met; these are artifacts, but very common), and number of missed cleavages allowed.

4. Matches are validated. Sequence coverage is typically more than 20%. Ascertain the number of matching peptides (at least four independent peptides), mass accuracy, and the number of peptides matching the protein versus the number of total peptides in the spectrum to avoid the interpretation of contaminated spectra. Criteria used for protein identifications and validation must follow the general guidelines for reporting proteomic experiments (MIAPE; http://www.psidev.info) (38).

3.8.3. Tandem Mass Spectrometry

1. Put the sample in the injection vial and inject using a precise injection method (i.e., one that delivers the exact amount with no sample loss).
2. Use a 60% B in 30 min HPLC gradient to analyze protein spots from 2D gels since there should be less than five different proteins in one spot.
3. The tandem mass spectrometry (MS/MS) data are acquired in the data-dependent mode allowing four to five precursors to be selected per survey scan and excluded for 60 s. Only doubly and triply charged peptides are allowed to be fragmented.
4. Parameters for the Q-TOF mass spectrometer are the following: positive mode, capillary voltage at 3.2 kV, a scan time of 1 s, and an inter scan delay of 0.1 s.
5. Spectra are processed with ProteinLynxGlobal server software 2.2. This allows background noise subtraction, smoothing, and deisotoping to generate a peak list file (.pkl). This is used to screen protein databases with the MASCOT search algorithm (version 2.2 Matrix Science) for searching against the National Center for Biotechnology Information nonredundant (NCBInr) database (taxonomy, Viridiplantae). The peptide tolerance is typically set to 0.2 Da and the MS/MS tolerance is set to 0.2 Da. Only doubly and triply charged peptides are searched for. A maximum number of one missed cleavage by trypsin is allowed, and carbamidomethylated Cys and oxidized Met are set as fixed and variable modifications, respectively. The MASCOT score cutoff value for a positive protein hit is set to the default value $p < 0.05$.
6. Protein hits are validated. To validate protein identification based on multiple peptides, only matches with individual ion scores above 20 are considered. In most cases, at least two different nonoverlapping peptide sequences of more than six amino acids with a mass tolerance <0.05 Da are conserved. Moreover, among the positive matches based on one unique peptide, only spectra containing a series of at least five consecutive y or b ions with individual ion scores above a threshold value calculated by the MASCOT algorithm with the used

search parameters are accepted (39). While more reliable results for peptide identification are obtained by MS/MS compared to PMF, validation criteria must be compliant with the MIAPE guidelines (38).

7. MS/MS spectra can also be interpreted de novo in cases in which there is poor database information for a given organism. Here, peptide sequence information can either be generated manually (PepSeq form Waters) or by several software programs (Peaks, ProteinLynx Global SERVER™). Validated sequences can be used with the MS-BLAST software to search for sequence homologies among the published protein sequences of other organisms. Manual de novo sequencing starts by searching in the low-mass range of the MS/MS spectrum for the presence of y1 ions, like Arg (m/z=175) or Lys (m/z=147), because the protein digestion has been done with trypsin (in which an Arg or a Lys residue is in the C-terminus position). The interpretation of the spectrum comprises extending the "y" ion series from low to high mass (up to a minimum of seven consecutive "y" ions) or the "b" ion series from high to low mass (up to a minimum of seven consecutive "b" ions) by considering the intensity of selected ions, mass error, the presence of immonium ions and the identification of H_2O (−18 Da) and NH_3 (−17 Da) losses. The signal intensity of other ion series (a, c, x, z) is often very low and difficult to annotate in the background (9).

4. Notes

1. The concentration of DMSO used does not have any effect on seed germination.
2. Store this solution in 10-mL aliquots at −20°C.
3. The freshly collected Cvi seeds are dormant, and the sterilization protocol has no discernable effects on seed dormancy.
4. In the storage conditions at 7°C and 40% RH, dormancy of Cvi seeds is maintained for around 1 year. The dormancy status of the seeds should always be checked before their use in experiments by a germination assay as described in Subheading "Seed Germination on Agar (For Studies with Dormant Seeds)."
5. In *Arabidopsis*, germination that ends with radicle protrusion through the covering layers (27–29) can be evaluated by following the two sequential steps of testa rupture and of endosperm rupture that is concomitant with root tip protrusion (30, 31).
6. The long-day photoperiod of the specified germination conditions gives the best expression of dormancy.

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Part V

Epigenetic Changes Associated with Seed Dormancy

Chapter 15

Chromatin Dynamics During Seed Dormancy

Yongxiu Liu, Regina Geyer, Vittoria Brambilla, Kazumi Nakabayashi, and Wim J.J. Soppe

Abstract

The chromatin structure determines gene expression and thereby regulates developmental processes in the plant. The molecular mechanisms regulating the induction and release of seed dormancy are still largely unknown and the underlying changes in chromatin organization have hardly been analyzed. Most chromatin studies in plants have been performed on vegetative tissues and have focused on seedlings. The composition of seeds hampers molecular analyses and requires adaptation of the methods that are used for other tissues. Here, we give an overview of the current methods that are used to study different aspects of chromatin organization in seeds. Cytogenetic methods, like fluorescence in situ hybridization and immunolocalization, are used to study chromatin at the microscopic level. Changes in DNA methylation and histone modifications can be studied with molecular methods, like bisulfite sequencing, immunoblotting, and chromatin immunoprecipitation.

Key words: Dormancy, Seeds, Chromatin organization, DNA methylation, Histone modifications

1. Introduction

All cells in an organism contain the same genetic information, but show differences in their spectrum of transcribed genes depending on the tissue and developmental phase. These differences are determined by the chromatin structure. Among the various developmental phases in the life cycle of a plant, the mature dry dormant seed represents a special case because of its very low metabolic and transcriptional activities.

Chromatin consists of a complex of DNA and proteins that constitute the chromosomes. The core particle of the chromatin is the nucleosome, which consists of DNA wrapped in 1.65 turns (147 bp) around a globular octamer made of two tetramers, each containing the histones H2A, H2B, H3, and H4. A spacer region

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exists in between two adjacent nucleosomes that can vary in length from 20 to 200 bp. A fifth histone, H1, can bind outside the nucleosome core (1).

The transcriptional activity of a gene depends on its accessibility to transcription factors, which is in turn dependent on the degree of condensation of chromatin. Chromatin condensation is determined by covalent modifications of specific amino acids at the N-terminal tails of the nucleosomal histones that face away from the interior of the core. These modifications include methylation, acetylation, ubiquitination, phosphorylation, ADP-ribosylation, and sumoylation and can occur in distinctly localized patterns. Generally, they are tightly regulated both developmentally and in a cell/tissue-specific manner because of their effect on transcription (2). Histone modifications can occur in various combinations and the different histone modification patterns form a "histone code" that extends the information of the genetic code (3). Different histone modifications can also depend on each other and these relationships, as well as the deciphering of the histone code, are still poorly understood.

The most studied histone modifications in plants are probably acetylation and methylation. Actively transcribed DNA is usually enriched in hyperacetylated histones compared to silent DNA. Histone methylation has differential effects on transcription, depending on the specific lysine that is methylated in the histone tail. The most studied are methylated histone H3 at lysine 4 and 36 (H3K4 and H3K36), which are associated with transcriptionally active chromatin structures and methylated H3K9, H3K27, and H4K20, which are associated with transcriptionally inactive chromatin structures. Antibodies for these and other modifications are commercially available. Histone modifications can be studied at individual genes by chromatin immunoprecipitation (ChIP). In this method, the material is first fixed with formaldehyde, which ensures cross-linking of protein and DNA. After shearing of the DNA, proteins together with cross-linked DNA are immunoprecipitated using antibodies against specific histone modifications. The cross-linking is subsequently reversed and DNA fragments are isolated and quantified usually by quantitative real-time PCR. The use of this method can give an overview of the distribution of a specific histone modification over a genomic region, for instance, a promoter region and coding region of a gene. In the last few years, this technique has been combined with tiling microarrays and high-throughput sequencing to obtain a genome-wide analysis of specific histone modifications (4, 5).

The DNA accessibility for transcription factors is also influenced by DNA methylation at the cytosine residue. Cytosine methylation can indirectly affect histone modifications and chromatin condensation. DNA methylation has a role in the transcriptional regulation

of genes, but is also important for the silencing of transposons. Transposons and other repeated sequences comprise most of the methylated DNA (6). The DNA methylation level of a specific gene or genomic region can be analyzed by digestion with methylation-sensitive restriction enzymes. The methylation level of every individual cytosine in a region can be obtained by bisulfite sequencing (7). Similar to the analysis of histone modifications, this technique has recently been combined with tiling microarrays and high-throughput sequencing to obtain a genome-wide overview of DNA methylation (8–11).

Chromatin organization not only influences transcription, but also replication, repair, recombination, and segregation of DNA. Furthermore, chromatin modifications determine the organization of DNA at a higher order (12). In *Arabidopsis*, the higher order chromatin structure can be visualized in the interphase nucleus by staining of DNA (Fig. 1a). Brightly stained regions (called chromocenters) consist of heterochromatin with compacted DNA while the rest of the nucleus has weaker stained euchromatin with less-compacted DNA. Euchromatin contains actively transcribed genes and heterochromatin contains centromeric and pericentromeric repetitive sequences and transposons (Fig. 1b). Heterochromatin and euchromatin are characterized by specific histone and DNA modifications (Fig. 1c, d); the DNA in chromocenters is, for instance, strongly methylated (13). The ratio between heterochromatin and euchromatin can be measured in isolated nuclei and by whole mount confocal microscopy, which also reveals the general size and shape of nuclei (14). The global levels of particular histone modifications at specific developmental stages or in specific tissues can be studied by immunoblotting using antibodies against a histone modification. The distribution of DNA and histone modifications in the nucleus can be visualized by immunolocalization (Fig. 1c, d), and the distribution of specific DNA sequences in the nucleus can be visualized by fluorescence in situ hybridization (FISH) (Fig. 1b) (15).

The changes in transcription that occur during seed maturation, after-ripening, and germination are likely to be reflected in changes of chromatin organization. However, the molecular mechanisms that regulate the induction and release of dormancy in seeds are still poorly understood and the chromatin organization in seeds is largely unknown. This lack of knowledge can be partially explained by the composition of seeds, which hampers molecular, biochemical, and cytogenetic analyses. The methods that are used to study chromatin structure in vegetative tissues require adjustments for successful application in seeds. The protocols that are described in this chapter are developed for *Arabidopsis*, but can also be applied to other plant species, although this might require specific modifications. Most of the protocols are still not optimal for seeds.

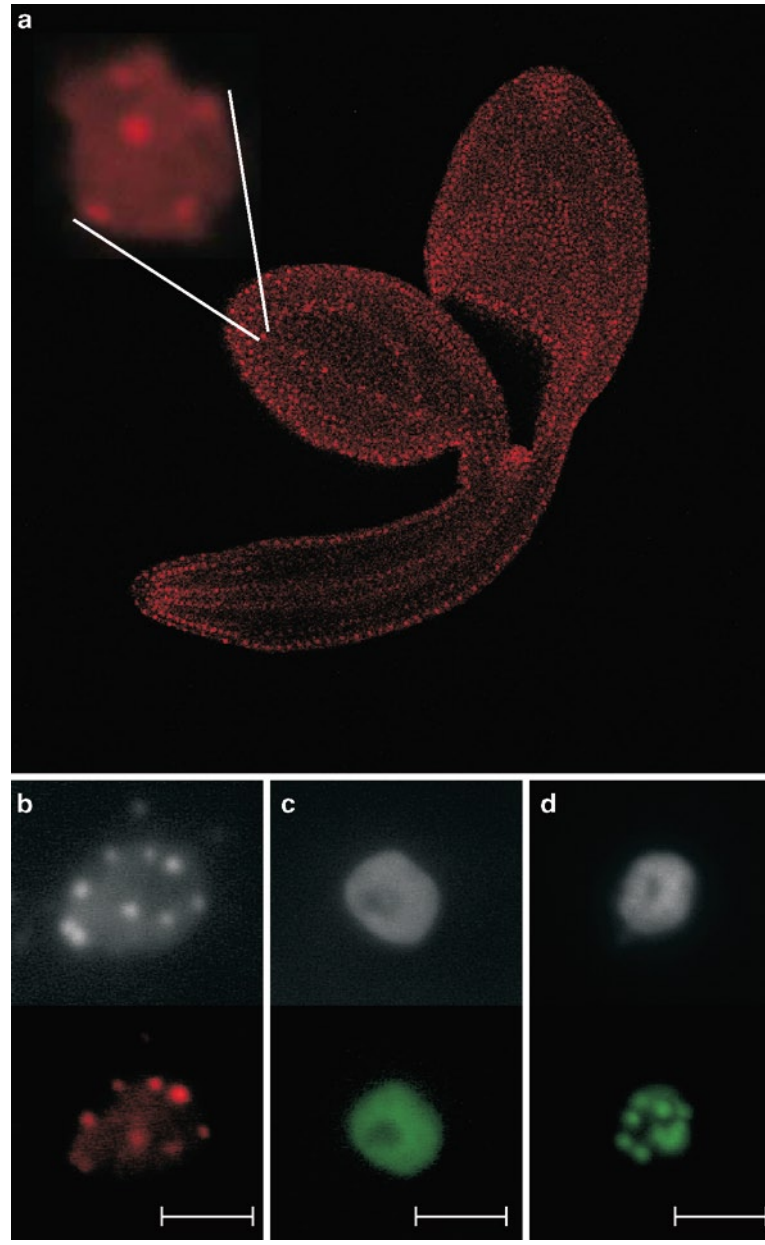

Fig. 1. Chromatin organization in *Arabidopsis* seeds. (**a**) Excised *Arabidopsis* embryo from a mature seed, whose DNA is stained with propidium iodide. The enlargement shows a single nucleus, chromocenters are visible as brightly stained spots. (**b**) DAPI-stained nucleus from a mature *Arabidopsis* embryo (*top*) and FISH signals on the same nucleus after probing with the 180 bp centromeric pAL repeat (visible in red, *bottom*). pAL repeat sequences localize in the chromocenters. (**c**) DAPI-stained nucleus from a mature *Arabidopsis* embryo (*top*) and fluorescent signals on the same nucleus after immunolabeling with antibodies against the euchromatic histone modification dimethyl H3K4 (visible in green, *bottom*). (**d**) DAPI-stained nucleus from a mature *Arabidopsis* embryo (*top*) and fluorescent signals on the same nucleus after immunolabeling with antibodies against the heterochromatic histone modification dimethyl H3K9 (visible in green, *bottom*). The appearance of the DAPI-stained nucleus in (**b**) is different from those in (**c**) and (**d**) due to different methods of nuclei preparation. Bar = 4 μm.

2. Materials

2.1. Cytogenetic Analysis of Interphase Nuclei

2.1.1. Measurement of Embryo Nuclei Volumes

1. Imaris 6.2.0 software (Bitplane, Zürich, Switzerland), including the Measurement Pro module to calculate image volumes.
2. Fix buffer: 1% formaldehyde, 10% DMSO, 2 mM EGTA pH 7.5, 0.1% Tween-20 in phosphate-buffered saline (PBS).
3. PBS: 0.038 M NaH_2PO_4, 0.162 M Na_2HPO_4, 1.5 M NaCl, adjust pH to 7.4.
4. PBT: 1% Tween-20 in PBS.
5. Enzymatic digestion mix: 0.5% (w/v) driselase, 0.5% (w/v) cellulase, 0.5% (w/v) pectolyase in 50 mM PIPES, 5 mM $MgCl_2$, 5 mM EGTA, pH 7.5.
6. RNAse: 100 µg/mL RNAse A, 1% Tween-20 in PBS.

2.1.2. Fluorescence In Situ Hybridization

1. Biotin-nick translation labeling kit (Roche, Indianapolis, USA).
2. Avidin conjugated with Texas Red and Goat anti-Avidin conjugated with biotin (Vector Laboratories, Burlingame, USA).
3. DAPI and Vectashield (Vector Laboratories).
4. Ethanol:acetic acid 3:1 (prepare fresh and keep on ice).
5. Citrate buffer: 10 mM Na citrate, pH 4.5 (store at room temperature).
6. Enzyme mix: 0.3% (w/v) pectolyase, 0.3% (w/v) cytohelicase, 0.3% (w/v) cellulase in citrate buffer (store at –20°C).
7. 20× SSC: 3.0 M NaCl, 0.3 M Na citrate, adjust pH to 7.0.
8. HB50: 2× SSC, 50% (v/v) deionized formamide, 5 mM Na phosphate, pH 7.0 (store at –20°C).
9. SF50: 2× SSC, pH 7.5, 50% (v/v) formamide in sterile deionized water (store at 4°C).
10. 4T: 4× SSC, pH 7.0, 0.05% (v/v) Tween-20.
11. 4M: 4× SSC, 5% (v/v) Boehringer blocking reagent (store aliquots at –20°C).
12. TNT: 100 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.05% (v/v) Tween-20.
13. TNB: 100 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.5% (v/v) Boehringer blocking reagent.

2.1.3. Immunolocalization on Isolated Nuclei

1. Rabbit polyclonal to histone H3 (di-methyl K4) (Abcam, Cambridge, UK) and Goat anti-Rabbit conjugated with Alexa-488 (Molecular Probes, Eugene, USA).
2. Nuclei isolation buffer (NIB): 10 mM HEPES, pH 7.5, 10 mM EDTA, 100 mM KCl, 0.5 M sucrose, 4 mM spermidine, 1 mM spermine.
3. 1% BSA in PBS.

2.2. Molecular Analysis of Chromatin Modifications

1. DNeasy Plant Mini Kit (Qiagen, Hilden, Germany).
2. Pellet paint co-precipitant (Novagen, Madison, USA).
3. EpiTect bisulfite kit (Qiagen).

2.2.1. Analysis of DNA Methylation with Bisulfite Sequencing

2.2.2. Immunoblot Analysis

1. Primary antibodies (specific to histone modifications) and secondary antibody (conjugated to horseradish peroxidase).
2. A luminescent detection system: Lumi-Imager detector (Boehringer Manheim).
3. Buffer A: 0.4 M sucrose, 10 mM Tris–HCl, pH 8.0, 10 mM $MgCl_2$, 0.1 mM PMSF, 5 mM 2-mercaptoethanol.
4. Buffer B: 0.25 M sucrose, 10 mM Tris–HCl, pH 8.0, 10 mM $MgCl_2$, 1% Triton X-100, 0.1 mM PMSF, 5 mM 2-mercaptoethanol.
5. Buffer C: 1.7 M sucrose, 10 mM Tris–HCl, pH 8.0, 2 mM $MgCl_2$, 0.15% Triton X-100, 0.1 mM PMSF, 5 mM 2-mercaptoethanol.
6. Separating buffer (4×): 1.5 M Tris–HCl, pH 8.8, 0.4% (w/v) SDS.
7. 30% acrylamide/bis-acrylamide (30:0.8) solution and *N*,*N*,*N*′,*N*′-Tetramethyl-ethylenediamine (TEMED).
8. Stacking buffer (4×): 0.5 M Tris–HCl, pH 6.8, 0.4% (w/v) SDS.
9. Running buffer: 25 mM Tris base, 192 mM glycine, 0.1% (w/v) SDS.
10. Laemmli buffer (2×): 125 mM Tris–HCl, pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 0.2 M DTT, 0.02% (w/v) bromophenol blue.
11. Prestained molecular weight markers: SeeBlue Plus2 Pre-stained standard (Invitrogen, Carlsbad, USA).
12. Blotting buffer: 100 mM Tris base, 192 mM glycine, 20% (v/v) methanol.
13. Ponceau S red solution: 0.5% Ponceau S in 1% acetic acid.
14. Blocking buffer: 5% (w/v) nonfat dry milk in TBS-T.
15. TBS-T: 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.05% (v/v) Tween-20.

2.2.3. ChIP of Nuclear Proteins from Dry Seeds

1. Protein A/G agarose coated with salmon sperm DNA.
2. Antibodies against specific nuclear proteins.
3. Fixation solution: 0.4 M sucrose, 10 mM Tris–HCl, pH 8.0, 1 mM EDTA, 1 mM PMSF, 1% formaldehyde.

4. NIB: 0.25 M sucrose, 15 mM PIPES, pH 6.8, 5 mM $MgCl_2$, 60 mM KCl, 15 mM NaCl, 1 mM $CaCl_2$, 0.9% Triton X-100, 1 mM PMSF, 1× Protease inhibitor cocktail for plant cells (Sigma, St. Louis, USA).
5. Lysis buffer: 50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% deoxycholate, 1% SDS, 1 mM PMSF, 1× Protease inhibitor cocktail for plant cells (Sigma).
6. Dilution buffer: 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris–HCl, pH 8.0, 167 mM NaCl, 0.1 mM PMSF, 1× Protease inhibitor cocktail for plant cells (Sigma).
7. Elution buffer: 1% SDS, 0.1 M $NaHCO_3$.
8. Low-salt wash buffer: 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl, pH 8.0.
9. High-salt wash buffer: 500 mM NaCl, 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl, pH 8.0.
10. LiCl wash buffer: 0.25 M LiCl, 1% NP-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris–HCl, pH 8.0.
11. TE wash buffer: 10 mM Tris–HCl, pH 8.0, 1 mM EDTA.

3. Methods

3.1. Cytogenetic Analysis of Interphase Nuclei

3.1.1. Measurement of Embryo Nuclei Volumes

1. Grow plants in standard long-day conditions (16-h photoperiod) (see Note 1).
2. Label 20–30 flowers per genotype at the day when pollination occurs (see Note 2).
3. Collect siliques of the required age, quickly remove the seeds, and fix them in the fix buffer in a 2-mL round-bottomed tube. Vacuum infiltrate for 10 min and incubate for 30 min (see Note 3).
4. Remove the fix buffer and wash in PBT for 3×10 min (see Note 4).
5. Incubate in 100% methanol for 2×2 min, in 100% ethanol for 2×2 min, and in xylene:ethanol (1:1) for 30 min.
6. Wash in 100% ethanol for 2×10 min (see Note 5).
7. Rehydrate samples by incubating in a series of ethanol dilutions (90, 70, 50, and 30% for 5 min each) and wash in PBS for 2×5 min.
8. Dissect embryos in PBS under a stereo microscope with a sharp-pointed tweezer and needles (see Note 6).
9. Incubate in enzymatic digestion mix for 30 min at 37°C and wash in PBS for 2×5 min.

10. Treat with RNAse for 1 h at 37°C and wash in PBS for 2 × 5 min.
11. Postfix in 1% formaldehyde in PBT and wash in PBS for 2 × 5 min (see Note 7).
12. Incubate for at least 15 min (maximum overnight) in 5 μg/mL propidium iodide and wash in PBS for 2 × 5 min.
13. Mount on microscopy slides with 0.2 μg/mL propidium iodide in Vectashield (see Note 8).
14. Observe samples under a confocal microscope.
15. Take images of at least 20 nuclei (5 per embryo) for each time point and genotype.
16. Take a series image of ten slices for a total stack depth of about 3–5 μm for each nucleus (see Notes 9 and 10).
17. Open the Imaris 6.2.0 program (see Note 11) and select "Open" in the menu.
18. Select the first image from the stack series to be analyzed and double click to open it (see Note 12). The image is displayed in the central Viewing Area.
19. Open the image as a Surpass Scene selecting "Surpass" from the upper toolbar. A 3-D image of the nucleus is visualized.
20. In the Objects toolbar of the Surpass view, click on the "add new Surfaces" icon.
21. Follow the Creation Wizard in the lower panel. Leave filters unselected in the Algorithm Settings. Select "Channel 1- Red" as Source Channel, "Absolute Intensity" as Thresholding, and "Manual 30.000" as Intensity Threshold and remove all filters in Classify Surfaces (see Note 13).
22. Check visually the quality of the surface construction in the central Viewing Area (see Note 14).
23. Click on the tab "Statistics" to start calculations and configure a list of visible statistical values.
24. Select "Volume" (see Note 15).
25. Copy the volumes data recorded for each sample into an excel file.
26. Calculate the standard deviation between independent biological replicates.

3.1.2. Fluorescence In Situ Hybridization

1. Imbibe seeds on wet filter paper (see Note 16), isolate the embryos, transfer them to a tube containing ice-cold freshly prepared ethanol:acetic acid (3:1), and store at –20°C.
2. Wash the fixed embryos in the tube with sterile deionized water for 2 × 5 min, followed by citrate buffer for 2 × 5 min.

Digest the embryos by incubating them in the enzyme mix in a closed tube at 37°C for at least 3 h.

3. Replace the enzyme mix with citrate buffer.
4. Place the digested embryos on a piece of parafilm and tap them with a blunt needle under the binocular until they become a homogeneous suspension (see Note 17). Transfer the suspension to a tube and place on ice (see Note 18).
5. Pipet 3–5 μL of the cell suspension on a slide and add 20 μL of 45% acetic acid with the help of a blunt needle. Place the slide on a heat block at 45°C and gently stir the drop with a blunt needle for 30–45 s (see Note 19).
6. Remove the acetic acid with drops of ice-cold ethanol:acetic acid (3:1), tilt the slide to remove liquid, and dry with a hair dryer (see Note 20).
7. Select slides and dry on a hot plate at 60°C for 30 min.
8. Pipet 80 μL of 100 μg/mL RNase (in 2× SSC) on a slide and add a coverslip (24 × 50 mm). Incubate the slides in a moist chamber at 37°C for 30–60 min.
9. Remove the coverslip and wash the slides in a container with 2× SSC for 2 × 5 min (see Note 21) and in PBS for 2 × 5 min.
10. Fix slides in 1% formaldehyde in PBS at room temperature for exactly 10 min and wash for 2 × 5 min in PBS.
11. Prepare the probe by mixing 4 μL of labeled DNA (see Note 22) with 6 μL of HB50 and 10 μL 20% dextran sulfate (dextran sulfate should have a final concentration of 10%). Vortex well and keep on ice.
12. Dehydrate the slides in an ethanol series of 70, 90, and 100% for 2 min each and dry the slides upright to prevent spots.
13. Add 20 μL of the probe directly on the slide, add a coverslip (24 × 24 mm), and denature the DNA on a hot plate at 80°C for EXACTLY 2 min.
14. Put the slides in a moist chamber and incubate overnight at 37°C.
15. Wash the slides in SF50 at 42°C for at least 3 × 5 min and in 2× SSC for 5 min (see Note 23).
16. Wash the slides in 4T for 5 min. Pipet 80 μL of 4 M buffer on the slides and add coverslips (24 × 50 mm) (see Note 24). Incubate in a moist chamber at 37°C for 30 min.
17. Remove coverslips and wash the slides in 4T for 5 min. Pipet 50 μL Avidin ~ Texas Red (dilute 1:400 in 4M) (see Note 25) on the slides and add coverslips (24 × 32 mm). Incubate in a moist chamber at 37°C for 30 min.

18. Remove coverslips and wash the slides in 4T for 2×5 min. Following a wash in TNT for 5 min, pipet 50 µL Goat anti-Avidin~Biotin (dilute 1:200 in TNB) on the slides, add coverslips (24×32 mm), and incubate in a moist chamber at 37°C for 30 min.
19. Wash in TNT for 3×5 min. Pipet 50 µL Avidin~Texas Red (dilute 1:400 in TNB) on the slides, add coverslips (24×32 mm), and incubate in a moist chamber at 37°C for 30 min.
20. Rinse in TNT for 3×5 min and dehydrate in an ethanol series of 70, 90, and 100% for 2 min each and dry.
21. Mount with 20 µL Vectashield+2 µg/mL DAPI. Add coverslips (24×24 mm) and store slides at 4°C. Signals can be detected with a fluorescence microscope.

3.1.3. Immunolocalization on Isolated Nuclei

1. Grind 150 mg seeds in a mortar with liquid nitrogen until the nitrogen has evaporated.
2. Add more liquid nitrogen and 1 mL NIB; grind this to a powder.
3. Wait until the powder becomes liquid and filter this suspension through a 50-µm mesh filter.
4. Fix the nuclei by adding an equal amount of 4% paraformaldehyde and keep on ice for 30 min.
5. Centrifuge the suspension at 1,260×*g* at 4°C for 10 min.
6. Resuspend the pellet in 600 µL NIB containing 0.3% Triton X-100.
7. Dilute a 2.3 M sucrose solution to 1.5 M with NIB and put this in a 1.5-mL tube.
8. Add the suspension on top of the sucrose solution and centrifuge at 12,000×*g* at 4°C for 10 min.
9. Wash the pellet twice in 1 mL NIB and centrifuge at 12,000×*g* at 4°C for 5 min after each wash.
10. Resuspend the pellet in about 50 µL NIB.
11. Pipet 3–5 µL on a clean slide and dry the droplet overnight at 4°C (see Note 26).
12. Select slides and postfix in 2% paraformaldehyde in PBS at room temperature for 30 min.
13. Wash slides in PBS for 2×5 min (see Note 21) and incubate in freshly prepared 0.5% Triton X-100 in PBS for 5 min.
14. Wash slides in PBS for 2×5 min. Pipet 100 µL 1% BSA in PBS, add coverslips (24×50 mm), and incubate in a moist chamber at 37°C for 30 min.
15. Wash slides in PBS for 5 min. Pipet 50 µL Rabbit anti-Histone H3K4 dimethylation (dilute 1:400 in 1% BSA in PBS) (see Note 27) on the slides, add coverslips (24×32 mm), and incubate in a moist chamber at 37°C for 30 min.

16. Wash slides in PBS for 3 × 5 min, followed by TNT for 1 × 5 min.
17. Pipet 50 µL Goat anti-Rabbit ~ Alexa-488 (dilute 1:100 in TNB) on the slides, add coverslips (24 × 32 mm), and incubate in a moist chamber at 37°C for 30 min.
18. Wash slides in TNT for 3 × 5 min, then dehydrate in an ethanol series of 70, 90, and 100% for 2 min each, and dry.
19. Mount with 20 µL Vectashield + 2 µg/mL DAPI. Add coverslips (24 × 24 mm) and store slides at 4°C. Signals can be detected with a fluorescence microscope.

3.2. Molecular Analysis of Chromatin Modifications

3.2.1. Analysis of DNA Methylation with Bisulfite Sequencing

Methylation of DNA can occur on cytosine residues. Bisulfite sequencing allows precise analysis of methylation by converting all nonmethylated cytosines into thymines while methylated cytosines remain unchanged.

1. Isolate DNA from siliques or seeds using the Qiagen DNeasy Plant Mini Kit or another DNA isolation kit (see Note 28).
2. Add sterile deionized water to 3 µg genomic DNA up to a final volume of 42 µL.
3. Add 5 µL of 10× reaction buffer (appropriate for restriction enzyme) and 3 µL restriction enzyme (~10 U/µg of genomic DNA) and incubate for 2–4 h at the appropriate temperature for the restriction enzyme (see Note 29).
4. Add sterile deionized water to the digested DNA to obtain a final volume of 300 µL.
5. Add 0.5 mL Tris-saturated phenol:chloroform:isoamylethanol (25:24:1) and vortex.
6. Centrifuge at top speed in a microfuge (~15,000 × *g*) for 2 min and transfer the upper aqueous phase to a clean 1.5-mL tube.
7. Add 0.5 mL of chloroform, vortex, and centrifuge at top speed for 2 min.
8. Transfer the upper aqueous phase to a clean 1.5-mL tube and add 0.1 volume of 3 M sodium acetate, pH 5.0, 3 volumes of 100% ethanol, and 2 µL pellet paint co-precipitant. Vortex the mixture.
9. Incubate overnight at –20°C.
10. Centrifuge at top speed at 4°C for 20 min, discard the supernatant, and add 1 mL 70% ethanol.
11. Centrifuge at top speed at 4°C for 2 min, discard the supernatant, and add 1 mL 70% ethanol.
12. Centrifuge at top speed at 4°C for 2 min, discard the supernatant, and air dry at room temperature for 10 min.

13. Dissolve the restricted DNA in 20 μL of sterile deionized water and store at –20°C.
14. Perform the bisulfite conversion using the EpiTect Bisulfite kit (see Note 30). All centrifugation steps are performed at room temperature.
15. Design primers and perform PCR reaction on the eluted, bisulfite-converted DNA (see Note 31).
16. Clone the PCR product into a plasmid and sequence the inserted PCR fragment (see Note 32).
17. Analyze the sequence data by comparison with the unmodified reference sequence (see Note 33).

3.2.2. Immunoblot Analysis

1. Grind 150 mg seeds in a mortar with liquid nitrogen until the liquid nitrogen has evaporated.
2. Add more liquid nitrogen and 1.5–2 mL of buffer A; grind this to a powder.
3. Wait until the powder becomes liquid and filter the suspension through a 100-μm mesh filter.
4. Centrifuge at 12,000 × *g* for 10 min and wash the pellet in 1.5 mL of buffer B.
5. Centrifuge at 12,000 × *g* for 10 min and wash the pellet in 0.8 mL of buffer C.
6. Layer this suspension over an equal volume of buffer C and centrifuge at 19,000 × *g* for 40 min.
7. Resuspend the pellet in 0.4 M H_2SO_4 and incubate on ice for 1 h with occasional mixing by hand.
8. Centrifuge at 8,000 × *g* for 5 min; take the supernatant and incubate on ice for 1 h with occasional mixing by hand.
9. Centrifuge at 8,000 × *g* for 5 min, take the supernatant, and precipitate protein in 15% trichloroacetic acid for 30 min on ice.
10. Centrifuge at 10,000 × *g* for 10 min and wash the pellet in 500 μL cold acetone.
11. Centrifuge at 10,000 × *g* for 10 min and repeat the acetone wash of the pellet.
12. Centrifuge at 10,000 × *g*, air dry, and resuspend the pellet in 50 μL 10 mM Tris–HCl, pH 8.0 (see Notes 34 and 35).
13. Prepare a 1.5-mm-thick, 15% mini gel by mixing 2.25 mL of 4× separating buffer, 4.5 mL of acrylamide stock solution, 2.25 mL of water, 30 μL of 10% ammonium persulfate solution, and 6 μL of TEMED. Pour the gel, and slowly cover the top of the gel with a layer of water or water-saturated isobutanol. Allow the gel to polymerize for 30–60 min (see Notes 36 and 37).

14. Pour off the water or water-saturated isobutanol and rinse the top of the gel with water.
15. Prepare the stacking gel solution by mixing 625 μL of 4× stacking buffer, 325 μL of acrylamide stock solution, 1.525 mL of water, 15 μL of 10% ammonium persulfate solution, and 3 μL of TEMED. Pour the gel and insert the comb. Allow the gel to polymerize for 30 min.
16. Carefully remove the comb, rinse the wells with running buffer, and set up the gel unit.
17. After adding running buffer to both chambers, apply protein samples (after adding Laemmli buffer) to the wells and include a prestained marker.
18. Run the gel with a constant current at 20 mA until the dye front reaches the bottom.
19. Disassemble the gel unit and remove unnecessary parts (e.g., the stacking gel). Equilibrate gel for 15–30 min in blotting buffer with gentle agitation.
20. Set up the semidry transfer stack. Place four sheets of 3MM filter paper saturated with blotting buffer on the anode. Place equilibrated nitrocellulose membrane (see Note 38) on top of the filter stack. Remove all air bubbles between membrane and filter paper by gently rolling a test tube or Pasteur pipette over the surface of the membrane (see Note 39).
21. Place the equilibrated gel on top of the membrane. Remove air bubbles again as described above.
22. Place another four sheets of filter paper on top of the stack and remove air bubbles in the stack.
23. Place the cathode on top of the transfer stack and electrotransfer at 0.8 mA/cm^2 of gel area for 1 h.
24. After transfer, disassemble the stack carefully and check if prestained markers are transferred.
25. Stain the blot in Ponceau S solution for 5 min at room temperature with gentle agitation. Rinse the blot in water and verify transfer efficiency (see Note 40).
26. Place the membrane in blocking buffer and rock gently for 1 h.
27. Incubate the blot for 1 h at room temperature with the primary antibodies (see Note 41).
28. Briefly rinse membrane with deionized water and wash in TBS-T for 2 × 20 min.
29. Incubate with the secondary antibodies conjugated with HRP for 1 h at room temperature (see Note 41).
30. Briefly rinse membrane with deionized water and wash three times in TBS-T for 10 min each.

31. Mix both reagents of the SuperSignal West Pico chemiluminescent substrate from Pierce (Rockford, IL) and add to the blot. Make sure that the reagent covers the blot and incubate for 2–3 min. Start signal detection using a luminescent detection system (see Notes 42 and 43).

3.2.3. ChIP of Nuclear Proteins from Dry Seeds

1. Immerse 500 mg of seeds in 30 mL fixation solution in a plastic flask and apply vacuum for 1 h (see Notes 44 and 45).
2. Stop the cross-linking reaction by adding glycine to a final concentration of 0.125 M (3.3 mL of 1.25 M glycine in 30 mL fixation buffer) and incubate for 10 min (see Note 46).
3. Remove as much of the solution as possible by placing the seeds on a filter on top of a paper towel. Rinse the flask with PBS and pour it over the seeds. Repeat the rinsing, allow the seeds to dry briefly, then flash freeze them in liquid nitrogen, and grind with a mortar and pestle.
4. Resuspend the ground seeds in 10 mL of NIB, mix, and incubate on ice for 10 min.
5. Centrifuge at 12,000 × *g* at 4°C for 20 min and resuspend the pellet in 1.2 mL of lysis buffer.
6. Sonicate 12 times for 10 s (see Note 47).
7. Centrifuge at 12,000 × *g* at 4°C for 5 min and transfer the supernatant to a new tube.
8. Add 40 μL of protein G agarose beads (salmon sperm DNA-coated), equilibrated in lysis buffer, to the supernatant and rotate for 1 h at 4°C (see Note 48).
9. Centrifuge for 30 s at 12,000 × *g* to pellet the beads (see Note 49).
10. Take a 200-μL aliquot from the supernatant, dilute 5× with dilution buffer, and store a 50-μL aliquot at –20°C. This serves as the input control (see Note 50).
11. Add for each immunoprecipitation (IP) 300 μL of the pooled supernatant to a tube with the appropriate antibody (see Note 51). Usually, about 10 μg of antibody should be used per IP.
12. Rotate overnight at 4°C (see Note 52).
13. Prepare 50 μL of protein G agarose beads (salmon sperm DNA coated) by rinsing three times with 1 mL of fresh dilution buffer in a 1.5-mL tube. Spin down for 5 s (see Note 53) between the washes to pellet the beads. Prepare one aliquot of beads per IP.
14. Add the IP to the tube with beads and rotate for 1–2 h at 4°C. In the meantime, prepare 10 mL of fresh elution buffer.
15. Centrifuge at 3,800 × *g* at 4°C for 30 s to collect the beads and discard the supernatant.
16. Add 1 mL of low-salt wash buffer per tube and rotate for 5 min at 4°C.

17. Centrifuge at 3,800×*g* at 4°C for 30 s to collect beads and discard the supernatant.
18. Add 1 mL of high-salt wash buffer per tube and rotate for 5 min at 4°C.
19. Centrifuge at 3,800×*g* at 4°C for 30 s to collect beads and discard the supernatant.
20. Add 1 mL of LiCl wash buffer per tube and rotate for 5 min at 4°C.
21. Centrifuge at 3,800×*g* at 4°C for 30 s to collect beads and discard the supernatant.
22. Add 1 mL of TE wash buffer per tube and rotate for 5 min at 4°C.
23. Centrifuge at 3,800×*g* at 4°C for 30 s to collect beads and discard the supernatant.
24. Repeat steps 22 and 23.
25. Elute the immune complexes by adding 270 μL of elution buffer. Vortex briefly to mix and incubate at 65°C for 15 min by reversing the tubes every 3 min. Centrifuge at 3,800×*g* for 2 min and transfer the supernatant to a fresh tube.
26. Repeat the elution and combine the two eluates.
27. Add 450 μL of elution buffer to the 50-μL input control of step 10 (to obtain a final volume of 500 μL).
28. Add 20 μL of 5 M NaCl to the samples and incubate overnight at 65°C (see Note 54).
29. Add 10 μL of 0.5 M EDTA, 20 μL of 1 M Tris–HCl, pH 6.5, and 2 μL of 20 mg/mL proteinase K per tube. Incubate for 1 h at 45°C.
30. Extract the samples with a PCR purification kit; the final elution volume should be 50–200 μL.
31. Perform real-time PCR analysis on the eluted templates with primers for your target gene to quantify the eluted DNA.

4. Notes

1. If different genotypes are compared, it is important to grow them at the same time since variation in growth conditions may affect seed maturation.
2. Label flowers that have just opened (white petals clearly visible) by tying a colored thread at the base of each flower.
3. All incubations should be at room temperature on a rocking plate.

4. Seeds should not dry out when solutions are changed.
5. At this point, samples can be stored at –20°C until use.
6. Breaking of the seed coat is sufficient and equally successful to complete dissection of the embryo.
7. At this point, samples can be stored for 1–2 weeks at 4°C.
8. Embryos are observed whole mount; when mounting the samples, avoid smashing of the sample.
9. The complete nucleus should be included in the stack.
10. When the images are taken, it is essential that the microscope settings are correctly adjusted and the lenses correctly selected for subsequent reliable volume calculation.
11. Measurement Pro module for statistical computations is required.
12. The system automatically loads all the images that belong to the selected data set.
13. The Surface Creation Wizard is divided into four steps, where different parameters can be adjusted. Click on the arrow to move from one step to another, then on the double arrow to finish, and start the statistical calculations. Further details on statistical formulas can be found in the Imaris 6.2.0 reference manual, which can be downloaded from the Web site http://www.bitplane.com.
14. Modifying the Manual Threshold level in step 3 of the Creation Wizard helps correcting differences in image brightness that might lead to an imprecise volume calculation.
15. The volume number represents the quantification of the space that the object within the surface occupies and is expressed in μm^3.
16. Seeds should not be imbibed for more than 2 h; otherwise, radicle emergence starts.
17. Take care that the suspension does not dry out.
18. The suspension can be stored up to a few hours at 4°C.
19. The nuclei are bound to the glass slide. It is useful to indicate the position of the nuclei with a marker because the suspension becomes nearly invisible after drying.
20. A number of slides can be prepared and stored for several months at room temperature.
21. All washing steps should be conducted at room temperature unless otherwise indicated.
22. Label a probe in advance with a Biotin-nick translation labeling kit according to the manufacturer's protocol.
23. If there is no time to continue with the detection, the slides can be stored in 2× SSC at 4°C.

24. The slides should not have any adherent drops that could dilute the added solution or antibodies.
25. This protocol is written for Texas Red detection, but alternative fluorescence systems can also be used (or mixed together in a single experiment). A frequently used system combines DIG-Nick translation-labeled probe with Mouse anti-Digoxigenin and Goat anti-Mouse ~ Alexa 488.
26. Slides can be stored for several months in the fridge.
27. This protocol gives dimethylated H3K4 as an example, which is a mark for active chromatin, but antibodies against other histone modifications in combination with appropriate secondary antibodies can also be used. The optimal dilution of the antibodies should be tested.
28. DNA isolation from dry seeds does not yield high amounts, and 200 × mg of siliques or 80 mg (divided in two portions) of freshly harvested dry seeds should be used to isolate about 3 μg of DNA. All buffer volumes have to be increased according to the amount of starting material. The obtained DNA quantity should be sufficient for the restriction step.
29. A restriction enzyme should be chosen which does not cut in the region of interest and results in fragments of about 2 kb. If necessary, a double digestion can be performed. It is important to use restriction enzymes that are insensitive to methylation.
30. Before starting the bisulfite conversion, read the EpiTect Bisulfite handbook carefully and prepare all the required solutions.
31. Primer selection is one of the most critical steps in bisulfite sequencing, and it is important that the primers contain as little CG as possible. METHPRIMER software (http://www.urogene.org/methprimer/index1.html) can be used for primer design. It is advisable to select several pairs of primers and to test them on bisulfite-modified DNA. The following two pairs of primers work very well on the *LINE1-4* and M*ULE* transposons and can be used as positive controls: *LINE1-4*, forward 5′-CTACAGTTTCTGAATCTCGAAGTCATTGAT-3′ and reverse 5′-TATAAAGAAGATCCTCATCACATGTCCATT-3′ and *MULE*, forward 5′-AAGGTGTTTCTATAAGCTCGGTAGTTGTTGTG-3′ and reverse 5′-CTTTCTCGGTACCTTCTCTTTAGGGAATTTTT-3′.
32. The PCR fragments can be cloned with the TOPO TA cloning kit (Invitrogen). After cloning, 10–20 colonies should be regrown for plasmid isolation and sequencing.
33. A convenient online tool for the sequence analysis is CyMATE (http://www.gmi.oeaw.ac.at/en/cymate-index/). Before analysis, the sequence should be aligned with the unmodified reference sequence.

34. Histone samples can be stored at −70°C until use.
35. Epitope-tagged histones can be affinity-purified when transgenic plants are available.
36. A sharp optical discontinuity at the overlay/gel interface is visible on polymerization.
37. Precast gels (e.g., Ready Gel Tris–HCl gel, linear gradient (BioRad, Hercules, USA)) can be used as well.
38. Nitrocellulose membrane should be floated in deionized water until completely wet, and then soaked in blotting buffer until use.
39. Be careful not to scratch the membrane surface.
40. Washing the blot with water eventually removes the red stain completely. Practically, it is not necessary to wash away the red stain completely before proceeding to a blocking step, since the stain disappears during blocking.
41. The appropriate dilutions of the primary and secondary antibodies are determined empirically.
42. The optimal time to detect the signal has to be determined empirically.
43. Sensitivity of detection of some proteins can be raised by autoclaving the membrane for 30 min before the immunodetection procedure (16).
44. This protocol is based on Oh et al. (17) and Saleh et al. (18).
45. It is essential that the seeds are fully immersed in the solution during the cross-linking step.
46. Vacuum is not necessary.
47. The time and number of pulses that have to be given vary depending on the sonicator and the required extent of cross-linking.
48. This step is performed to clean the supernatant.
49. Sonicated chromatin can be frozen at −80°C for 3 months without significant loss of chromatin quality or can be used directly for immunoprecipitation.
50. A 10-μL aliquot of the pooled supernatant can be used to test the sonication efficiency by DNA gel electrophoresis.
51. A control, in which no antibody is added to the supernatant, should also be performed and further processed with the IP.
52. This protocol can be divided over 3 days. Steps 1–12 take place on the first day, steps 12–28 on the second day, and steps 28–31 on the third day.
53. The beads should not be centrifuged at a higher speed than 3,800 × *g*.
54. The incubation should last at least for 6 h.

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Chapter 16

Emerging Mass Spectrometry-Based Technologies for Analyses of Chromatin Changes: Analysis of Histones and Histone Modifications

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Abstract

Mass spectrometry (MS) is rapidly becoming an indispensable tool for the analysis of posttranslational modifications (PTMs) of proteins, and particularly histone PTMs that regulate physiological processes. The more traditional *bottom-up* approach of searching for modifications on peptides rather than intact proteins (*top-down*) has proven useful for finding phosphorylation, acetylation, and ubiquitination sites. With the use of modern instrumentation and various MS-based techniques, peptides and their PTMs can be characterized in a high-throughput manner while still maintaining high sensitivity and specificity. In complement to bottom-up MS, recent advances in MS technology, such as high-field Fourier transform ion cyclotron resonance (FTICR)-mass spectrometry, have permitted the study of intact proteins and their modifications. On-line and off-line protein separation instruments coupled to FTICR-MS allow the characterization of PTMs previously undetectable with bottom-up approaches. The use of unique fragmentation techniques in FTICR-MS provides a viable option for the study of labile modifications. In this chapter, we provide a detailed description of the analytical tools – mass spectrometry in particular – that are used to characterize modifications on peptides and proteins. We also examine the applicability of these mass spectrometric techniques to the study of PTMs on histones via both the bottom-up and top-down proteomics approaches.

Key words: Histones, Mass spectrometry, Top-down proteomics, FTICR-MS, Acetylation, Posttranslational modification, Chromatin, Epigenetics

1. Introduction

1.1. Definition and Function of Histones

Histones are small protein (100–200 amino acids) whose sequence and function are highly conserved throughout eukaryotes. The main function of histones is to form the structural components of chromatin and chromosomes; however, they also commonly play a

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role in regulating transcription and repairing DNA. Although histones are conserved, the different types can be distinguished by molecular weight, amino acid composition, amino acid sequence, activity, and function. By convention, human histones are divided into five classes: H1, H2A, H2B, H3, and H4. While H1 is a linker protein, the latter four histones form an octameric core around 146 base pairs of DNA. The core histones also leave an unstructured amino-terminal tail domain consisting of about 25–40 residues as exposed sites for posttranslational modification (PTM) (1).

PTMs are an integral part of the regulation of histone activity (2). Acetylation is the process of adding acetyl groups to a chemical compound, often to hydroxyl and amine groups. In proteins, N-acetylation commonly occurs on exposed N-termini; N-acetylation of lysine residues on the histone tail neutralizes its positive charge, thereby reducing its affinity for DNA and allowing for higher levels of transcription (3). Ubiquitination also modifies the lysine residues of histones and can have a similar effect on transcriptional regulation (4, 5). In contrast, phosphorylation typically occurs on serine, tyrosine, and threonine residues of the histones. In particular, serine residues in histones have long been known to be phosphorylated. Ser 10 in histone H3 is one such case. The addition of a phosphoryl group at this site activates a DNA repair mechanism and has been correlated with gene activation (6). The phosphorylation of other serine residues has similar downstream effects while the inability to add a phosphoryl group to these residues has been implicated in a number of human disorders (2).

1.2. Mass Spectrometry as a Tool for the Study of Histones

PTMs on histones have been extensively studied using mass spectrometry (MS)-based methods. Mass spectrometry is an analytical technique that allows for accurate molecular characterization of PTMs on proteins. The general layout of mass spectrometers consists of: an *ionization source* (which converts the analyte into charged particles and transfers the analyte into the gas phase); *a mass analyzer* (which separates the charged ions according to their mass-to-charge ratio, m/z); and a *detector* (which detects the separated charged ions). Two of the most common types of ionization sources that are used in the analysis of PTMs are matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI) (7).

In MALDI, the sample is co-crystallized with a light-absorbing organic compound (a matrix) on a solid metal surface. A laser beam is then directed at the spot containing the matrix and analyte molecules, and the photoexcited matrix molecules assist in the desorption of ionized analytes. For peptides, in most cases, these ions are singly charged and are subsequently separated by the mass analyzer (Fig. 1) (8). ESI uses a small, charged metal capillary at high voltage through which a volatile solvent containing the analyte is forced to form a fine spray. After the formation of the aerosol, the volatile solvent begins to evaporate, forcing the analyte

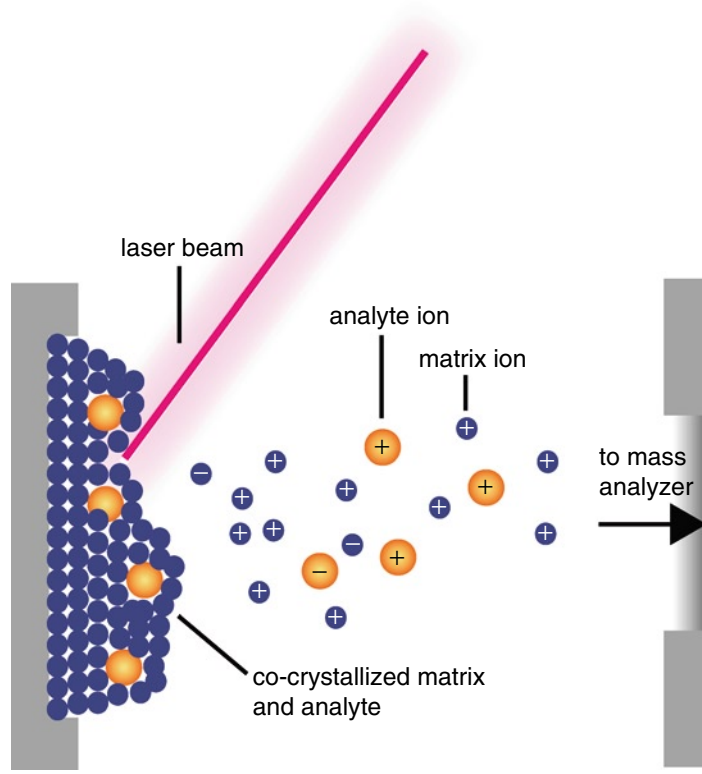


Fig. 1. Matrix-Assisted Laser Desorption/Ionization (MALDI). The sample containing analyte molecules is mixed with matrix solution and a small volume (often 1 μl) is spotted onto an MALDI target plate. The matrix solution contains a UV-absorbing compound in a mixture of water and volatile organic solvents. The organic solvent aids in solubilizing hydrophobic analyte molecules while water does the same for hydrophilic analytes. As the solvent evaporates, the matrix and analyte co-crystallize. A UV laser is then directed at the sample spot and the matrix crystals rapidly absorb the radiation. The matrix assists the desorption of the analyte off the target plate into the gas phase. This step protects the analyte from degradation by the laser. The matrix and analyte molecules are now ions consisting of the original molecule and a proton [$M+H^+$]. With the application of an electric field, they can be accelerated into the mass analyzer. MALDI most often creates singly-charged ions for peptides and small molecules.

ions closer together. This creates strong repulsive forces that drive the ions apart in a process termed Coulomb fission. The combination of Coulomb fission and solvent evaporation produces dehydrated ions (9), which are then directed into the mass analyzer (Fig. 2). MALDI-MS and ESI-MS are referred to as "soft" ionization methods because they result in less fragmentation of the analyte than other ionization techniques, such as electron impact. MALDI and ESI dominate the current analysis of protein PTMs largely due to their ability to form charged molecules while keeping the analyte intact during the ionization process, even for relatively labile modifications, like phosphorylation.

Commonly used mass analyzers include the time-of-flight (TOF), quadrupole, and ion trap. In a TOF analyzer, ions are

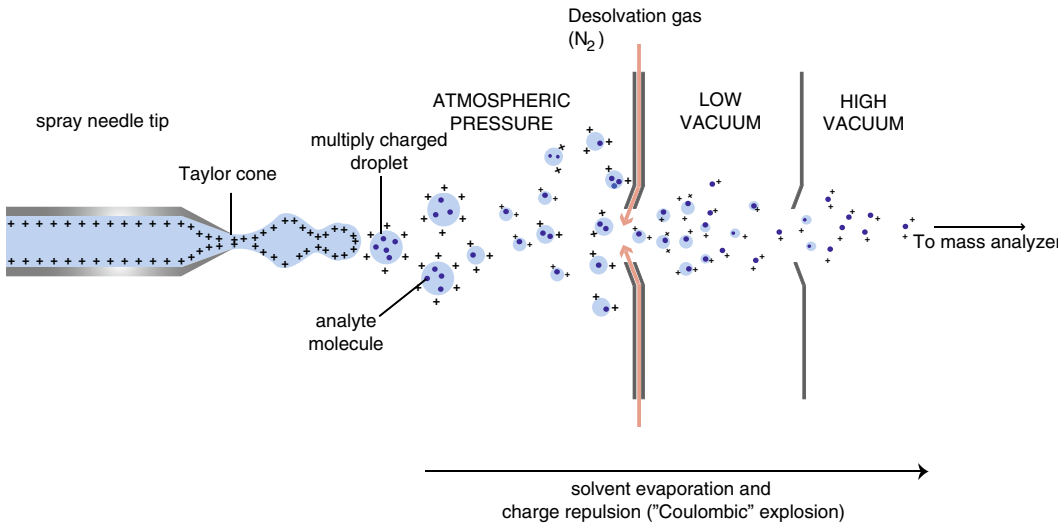


Fig. 2. Electrospray ionization (ESI). A mixture of analytes dissolved in a volatile solvent is ejected out of a small, charged, metal capillary to produce an aerosol spray in a conical shape, termed a Taylor cone. The aerosol produced at atmospheric pressure is introduced into the mass spectrometer, where sequentially a vacuum is employed. This causes evaporation of the volatile solvent forcing the charged analytes to come closer together; eventually, they repel one another by a process called Coulomb fission. The combination of solvent evaporation and Coulombic fission repeats until the analyte molecules exist as lone ions free of solvent. These ions are then accelerated into the mass analyzer of the mass spectrometer. Unlike MALDI, it is common for ESI to generate multiply charged ions for peptides.

accelerated into a field-free region and ultimately strike the detector. As the name, TOF, suggests, the amount of time that the ion takes to strike the detector is directly proportional to the mass of the ion (10–12). Another popular mass analyzer is a quadrupole (Fig. 3). This instrumental setup employs four parallel rods that emit radio frequency (RF) and direct current (DC) voltages to guide the ions. Unwanted ions are destabilized so that they do not reach the detector while ions of interest are stabilized and permitted to pass through the quadrupole. Setting these voltages to a fixed value or scanning these voltages allows selective or nonselective transmission of ions, respectively (13, 14). This is in contrast to a TOF, which does not scan and therefore cannot discriminate and detects all ions. In an ion trap mass analyzer, ions are injected into and trapped in a chamber, where specific ions of interest can be subsequently selected for fragmentation, and unwanted ions can be ejected (15, 16). The ability of quadrupole and ion trap analyzers to select or *gate* peptides has been proven to be very useful for sample analysis via MS, especially by a method termed tandem mass spectrometry (MS/MS) (described below) (17). All of these mass analyzers are suitable for use with either of the aforementioned common ionization sources (Fig. 4).

After the ions are separated in the mass analyzer, the ions reach the detector. Most modern mass spectrometers contain detectors that generate an electrical signal from the collision of the incident

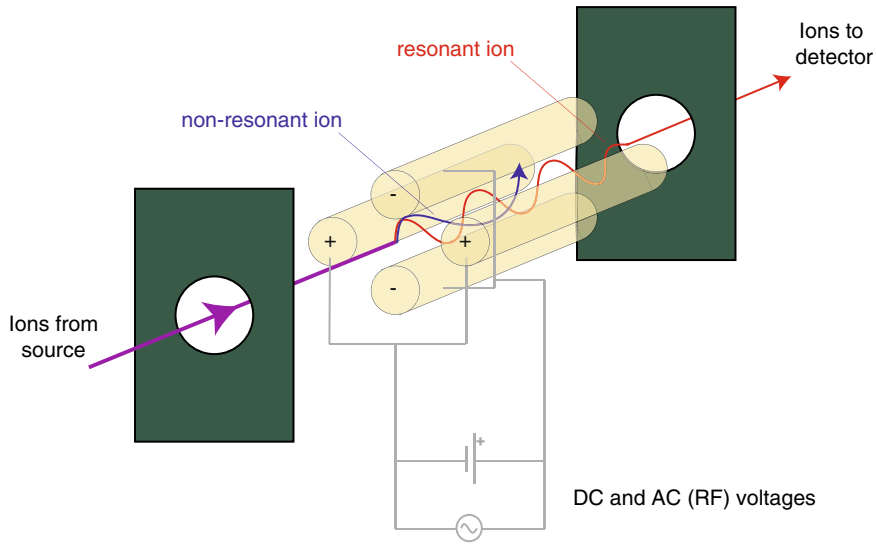


Fig. 3. Quadrupole mass analyzer. Four metal rods are arranged such that opposing rods are electrically connected for the application of direct current (DC) and radio frequency (RF) potentials. Ions from an ionization source, such as MALDI or ESI, are subjected to the voltages as they enter the quadrupole and, depending on the ratio of DC to RF potentials, only ions with a certain mass-to-charge ratio pass through while other ions are destabilized. The selected ions then exit the quadrupole toward the downstream detector.

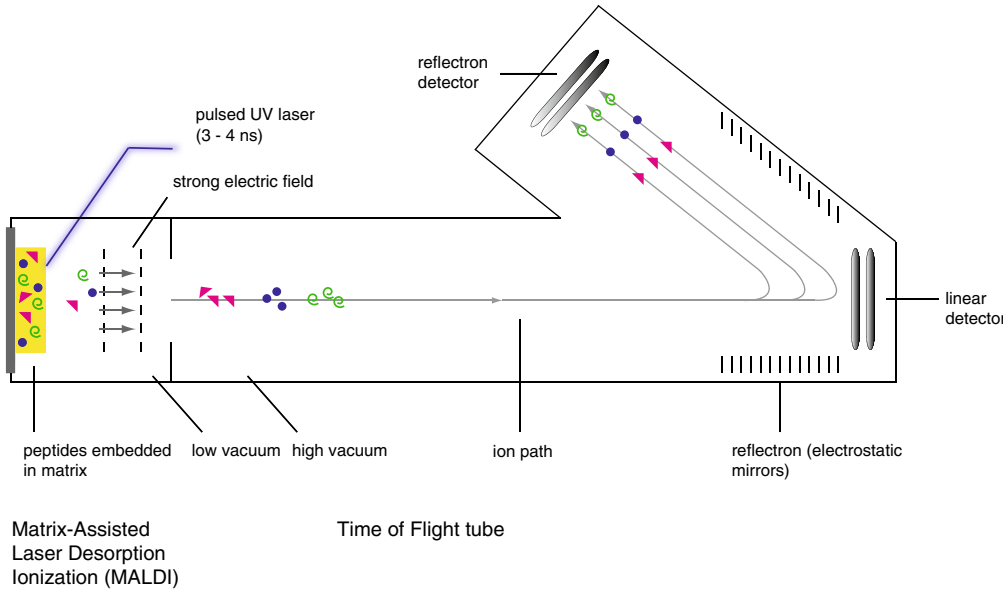


Fig. 4. MALDI-TOF mass spectrometer. The process of MALDI creates ions that are moved into the mass spectrometer by applying a strong electrical field. The ions travel through the field-free time-of-flight tube, where they are separated based on their mass-to-charge ratios. Heavier ions travel more slowly than lighter ions. The ions then hit the linear detector or, if reflectron mode is enabled, they will travel further to strike the reflectron detector. In reflectron mode, a static electric field is applied near the location of the linear detector so that ions with the same mass, but slightly different kinetic energies and therefore different velocities, arrive at the reflectron detector at the same time. In this manner, the reflectron enables the improvement of mass resolution.

ion beam on a resistive conductive surface (18). For instance, an electron multiplier (EM) device uses a process called secondary electron emission to amplify the signal from the original ions (19). Another type of detection method, cyclotron frequency detection, is used by Fourier transform ion cyclotron resonance (FTICR)-mass spectrometry. This involves the induction of an ion image current through the generation of an electrical signal (20).

In a quadrupole FTICR-MS instrument, the analytes exit the ion transfer capillary and travel through a series of ion guiding and focusing devices, a mass-prefilter quadrupole, a mass-selective quadrupole and a collision-cell hexapole. After passing through the ion transfer optics, the analytes eventually reach the ICR cell, where they are trapped, excited, and then detected (Fig. 5). In the ICR cell, the analytes are under the influence of a magnetic field. The orientation of the magnetic field when combined with excitation RF pulses, *chirps*, causes the analytes to move in cyclotron orbits proportional to their individual mass-to-charge ratios. Instead of hitting the detector, ions with the same *m/z* pass close to a pair of the detector plates at a unique cyclotron frequency which is proportional to the mass and charge (21). For every ion that passes by, an image current is generated. The sum of all of these currents makes up the convoluted free induction decay (FID) signal (20, 22). This FID signal is deconvoluted by applying a mathematical operation called the Fourier transform. This converts the FID signal into an interpretable frequency spectrum, which is then converted to the final interpretable mass spectrum after calibration with standard compounds (Fig. 6) (22). Like the previously-mentioned mass analyzers, FTICR-MS can utilize either MALDI or ESI as the ionization source.

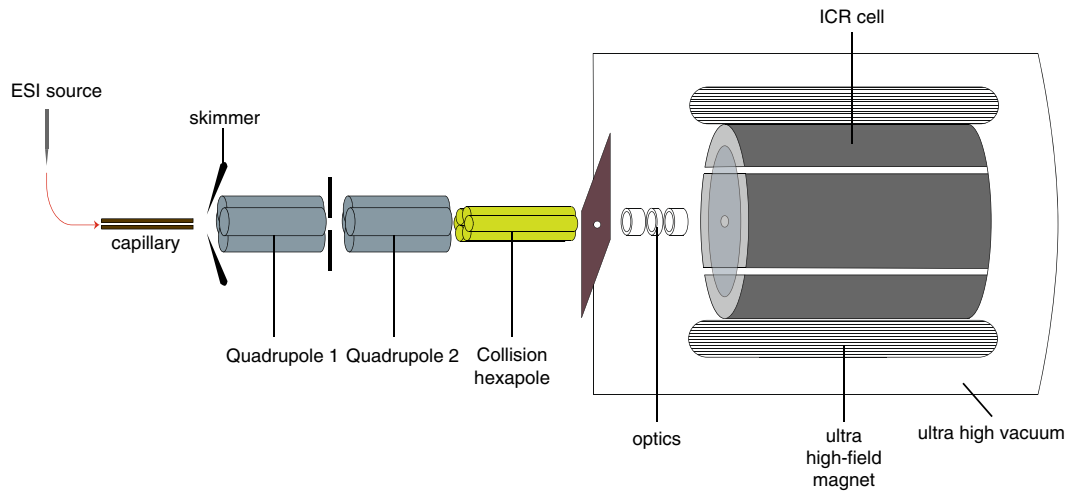


Fig. 5. Qq-FTICR-MS. Ions enter the mass spectrometer and travel through a series of quadrupoles for ion guiding and focusing electronic devices, a mass-prefilter quadrupole, a mass-selective quadrupoles (Q), and a collision-cell hexapole (q) where the quadrupole-selected ions are fragmented. The ion transfer optics guide the resulting ion beam into the ICR cell for trapping, excitation, and detection.

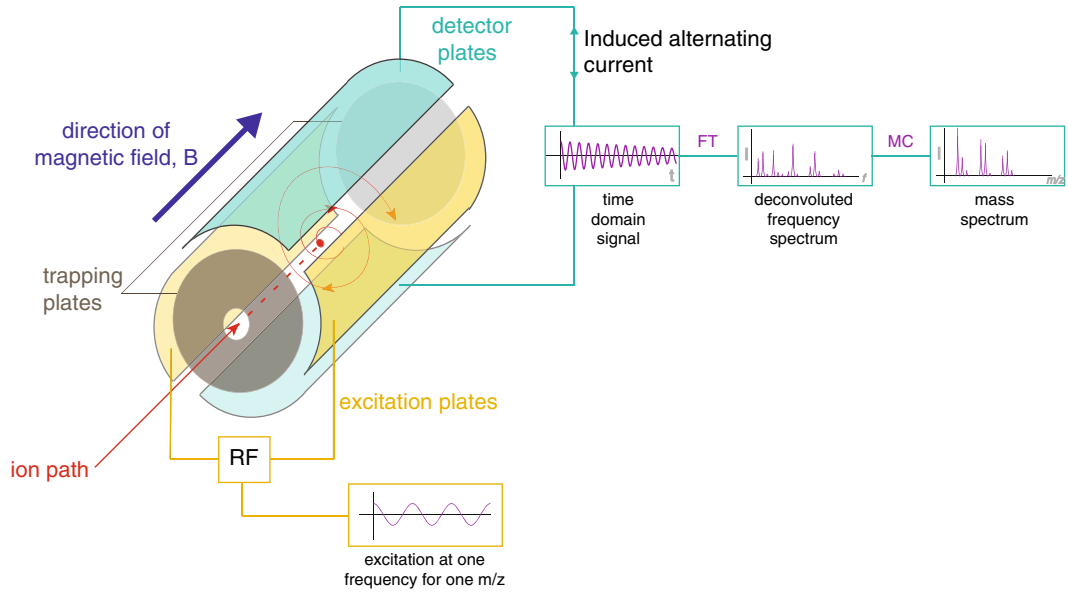


Fig. 6. Ion cyclotron resonance (ICR) cell. As the ions enter the ICR cell, voltages are applied at the front and back trapping plates in order to retain the ions in the ICR cell. An excitation pulse ("chirp") is applied to the trapped ions. Under the influence of the magnetic field, the ions travel in an epitrochoid orbit. As the ions pass by the detector plates, an image current is generated and detected by the detector plate. The free induction decay (FID) signal is generated as the sum of image currents generated by all the ions in the ICR cell. This signal is deconvoluted by applying a Fourier transform to yield the final mass spectrum, after calibration with the reference masses of standard compounds.

Although MS is able to detect mass shifts of analytes due to modifications, determination of the exact residue within a protein or peptide that is modified requires a different approach termed *tandem MS* or *MS/MS*. MS/MS involves the fragmentation of a mass-selected molecule of interest. Fragmentation is achieved through various means, such as collision of the molecules with inert gas (termed collision-induced dissociation, CID or collisionally activated dissociation, CAD) or electrons (termed electron-capture dissociation, ECD) (7). A dissociation method similar to ECD is electron-transfer dissociation (ETD). However, in the case of ETD, dissociation of the analyte involves the transfer of charge by a radical anion rather than an electron (23). Alternatively, infrared multiphoton dissociation (IRMPD) is a fragmentation method in which ions absorb infrared photons emitted by a CO_2 laser (24). IRMPD, ECD, and ETD are most often used with FTICR-MS. ECD and ETD are particularly advantageous for studying PTMs on proteins because, unlike other dissociation methods, they leave the modifications intact and bound to the residue while fragmentation can still occur along the peptide backbone (23, 25).

All of these dissociation techniques can yield different, but predictable, fragments which are named using Roepstorff nomenclature (Fig. 7) (26). For example, the y-ion and b-ion series result from cleavage between the CO and the NH of the peptide backbone. The b_1 ion includes the N-terminal amino acid while the b_2

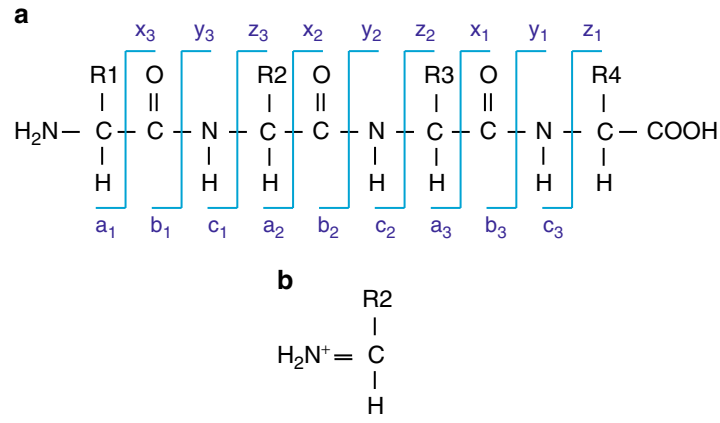


Fig. 7. Peptide fragmentation nomenclature. (**a**) Roepstroff nomenclature for naming the fragment ions obtained by collision within the mass spectrometer. (**b**) Fragmentation can also create internal cleavage ions, such as immonium ions. The immonium ion is the combination of an "a type" and "y type" fragmentation.

ion includes the first two N-terminal amino acids of the peptide. The corresponding y_1 ion contains the C-terminal amino acid while the y_2 ion contains the two C-terminal amino acids of the peptide. The a-ion and x-ion series are the naming systems for fragmentation that occurs between the α-carbon and the carboxy carbon of amino acid residues of a peptide. The c-ion and z-ion series are the naming systems for fragmentation that occurs between the amido-nitrogen and the α-carbon of the amino acid residues of a peptide. Fragmentation can also give rise to internal cleavage ions, which result from cleavage at *two* sites along the peptide backbone. For example, immonium ions are those created by an "a type" and "y type" fragmentation (see Fig. 7).

1.2.1. Liquid Chromatography-Mass Spectrometry

Liquid chromatography (LC) using various chromatographic columns and solvents can be coupled to MS (LC/MS) to separate a complex mixture of analytes prior to mass spectrometric analysis. In the LC, analytes interact with the stationary-phase material in the column where they are retained and eluted based on their physical properties, such as hydrophobicity. The end of the column is connected on-line to the MS source. As the separation progresses, analytes are eluted out of the LC column and introduced sequentially into the MS source. Because of this additional online separation, LC reduces one of the major problems of ESI-MS when analyzing a highly-complex mixture: the suppression of signal of peptides due to the presence of other peptides in the source at the same time. This phenomenon of ion suppression is particularly problematic for low-abundance and phosphorylated peptides. Since most MS methods operate in the positive ion mode (i.e., positive ions are detected), the signal from the negatively-charged phosphoryl group often decreases the detection sensitivity of a

phosphopeptide (27). A trapping column with a hydrophobic stationary phase may be used in conjunction with the online LC to remove salts and to preconcentrate the sample prior to injection.

1.2.2. MS Scan Modes

In addition to LC-MS, specific MS/MS scans, like *product ion* or *precursor ion scanning*, can be performed to analyze PTMs. Product ion scanning is one of the conventional types of MS scans. Operationally, the MS mode (survey scan) is first used to determine the m/z of the peptides, and these m/z values (the precursor ions) are then selected for fragmentation in the collision cell forming product ions. These product ions are subjected to a second m/z-based separation. These two m/z-based separation steps give rise to the term MS/MS. From the product ion spectra, the sequence of a peptide as well as the position of the modification can often be determined.

Many posttranslationally-modified peptides yield MS/MS spectra that contain unique fragment ion characteristic of the modifying group. In these instances, precursor ion scanning can be used to determine whether a peptide contains a specific PTM. This type of scanning is typically performed on a triple quadrupole mass spectrometer, which has multiple quadrupoles aligned in tandem. It can be used to confirm the modification of a precursor ion (i.e., peptide) that results in a modification-specific product ion. When a complex mixture of peptides enters the mass spectrometer, the first quadrupole scans all of the precursor ions in the sample. The second quadrupole fragments these transmitted precursor ions using CID/CAD to yield product ions. Only a specific product ion of selected m/z is gated by the third quadrupole. This results in a spectrum of precursor ions that yield the selected product ion (Fig. 8). Due to the nature of this method, background noise is diminished because fewer ion signals are present in the spectrum (28). This renders the mass spectrum easier to interpret (28, 29).

Precursor ion scanning using a Q-TOF mass spectrometer is different from conventional precursor ion scanning. Rather than having several transmission quadrupoles, a Q-TOF has at least one quadrupole followed by a TOF analyzer. Because of the nature of the TOF, it cannot discriminate between ions to allow only one ion through. Instead, all generated product ions are detected and their m/z values recorded. Post-processing software is then used to determine which precursor ions yielded the product ion of interest. The advantage of using a Q-TOF over a triple-quadrupole or ion-trap mass spectrometer is that multiple precursor ion scans (for multiple product ions) can be created from one data set. However, the sensitivity is somewhat lower when using a Q-TOF mass spectrometer (30). Nonetheless, precursor ion scanning of modified peptides has proved useful for the identification of chemical and PTMs on proteins of interest in a more complex mixture (28).

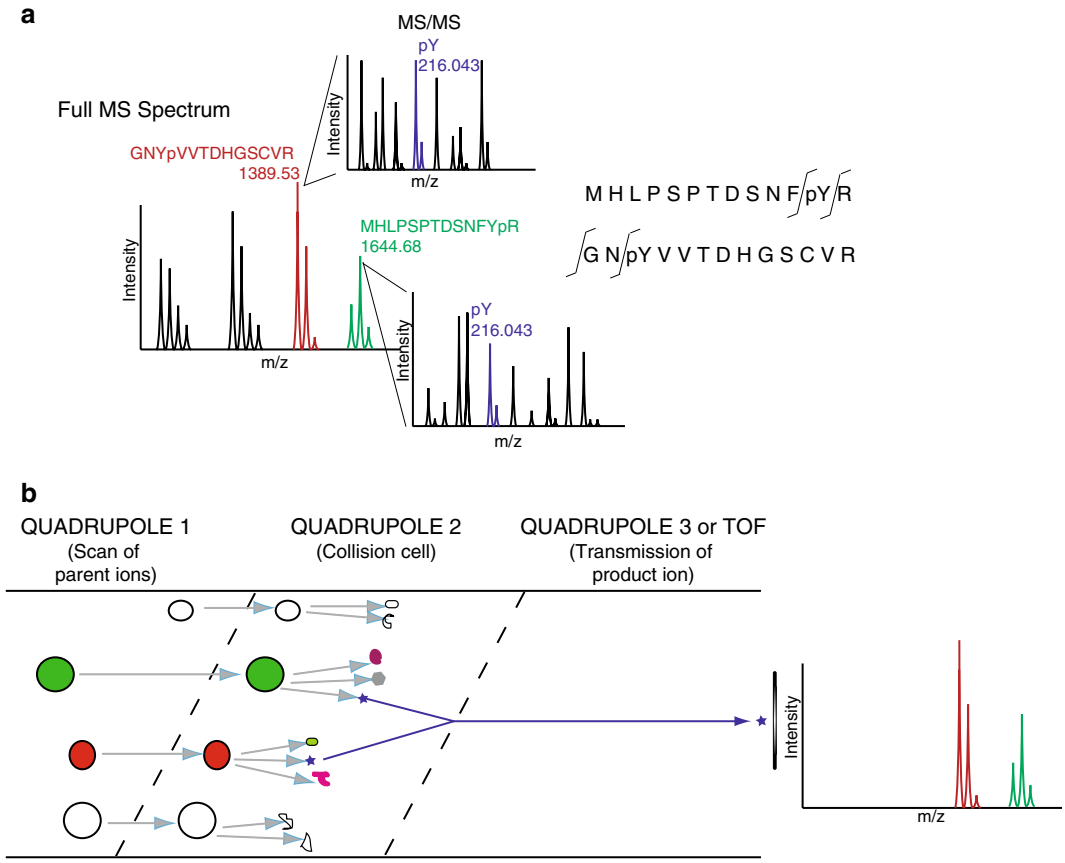


Fig. 8. Precursor ion scanning. (**a**) An MS scan mode for a complex peptide mixture yields a spectrum in the format of the "Full Mass Spectrum." Inset, MS/MS of two analytes in the spectrum shows that these two peptides in a complex mixture yield the same phosphorylated tyrosine immonium ion at *m/z* 216.043. (**b**) In precursor ion scanning mode, the first quadrupole is set to scan all ions in a complex mixture. The second quadrupole acts as a collision cell and fragments every ion coming from the first quadrupole. The third quadrupole is set to scan for the *m/z* of only one product ion (i.e., 216.043). All precursor ions which yield that fragment ion will be detected. Note the difference between the MS spectrum of the sample complex peptide mixture in MS mode (**a**) and in precursor ion scanning mode (**b**).

1.2.3. Bottom-Up and Top-Down Proteomics

With the use of MS and MS/MS, biological molecules can be effectively studied and characterized to ascertain the types and locations of the PTMs. In general, there are two approaches to studying PTMs on proteins. The traditional approach is known as *bottom-up* proteomics, where the target protein is proteolytically digested into peptides prior to MS and MS/MS analysis. The alternative technique is known as the *top-down* approach. This involves the ionization of an intact protein and its dissociation into fragments while in the gas phase inside the mass spectrometer. In both approaches, the masses of the dissociation products enable the identification and location of PTMs on proteins. Top-down proteomics, however, can provide information about protein isomers and stoichiometry of PTMs. Top-down

approaches have become more feasible in recent years with improved instrumentation (i.e., FTICR-MS) and software (i.e., ProSight PTM 2.0) (31–34).

2. Materials

2.1. In-Solution Digestion of Proteins

1. Water, HPLC grade.
2. Trypsin, sequence grade (Promega, Madison, WI).
3. Glu C.
4. Ammonium bicarbonate.
5. Low-retention micro-centrifuge tubes (Axygen, Union City, CA).

2.2. MALDI-MS and MS/MS

1. Cyano-4-hydroxycinnamic acid (CHCA) (Sigma–Aldrich, St. Louis, MO).
2. Ammonium citrate.
3. HPLC-grade acetonitrile.
4. Trifluoroacetic acid (TFA).
5. 4700 Proteomics Analyzer Mass Standards Kit – contains des-Arg1-Bradykinin, Angiotensin, Glu1-Fibrinopeptide, and three ACTH peptides (AB Sciex, Concord, ON, Canada).
6. 4800 MALDI-TOF/TOF with target plate (AB Sciex).

2.3. Immobilized Metal Ion Affinity Chromatography

1. Ni-NTA resin (nickel-bound nitrilotriacetic, a quadradentate metal chelator) (Qiagen, Chatsworth, CA).
2. HiTrap™ Chelating HP column (1 mL or 5 mL), consisting of iminodiacetic acid (IDA, a tridentate metal chelator) bound to highly cross-linked agarose beads (Pharmacia Biotech, Piscataway, NJ).
3. Compact Reaction Columns (USB Inc, Cleveland, Ohio).
4. EDTA, acetic acid, $GaCl_3$, and $FeCl_3$.
5. HPLC-grade water.

2.4. On-Target Dephosphorylation

1. Alkaline phosphatase (PPase) (New England Biolabs, Ipswich, MA).
2. Ammonium bicarbonate.
3. Phosphoric acid.

2.5. Acetylation of Lysine ε-Amino and N-terminal Amino Groups

1. Ammonium bicarbonate.
2. Acetic anhydride.
3. Ammonia.
4. Methanol (MS grade).

5. Water (MS grade).
6. C18 Sep-Pak™ cartridge.

2.6. Two-Step Acetylation/Tri-Deuteroacetylation of Sample Protein

1. Acetic anhydride.
2. Hexadeuteroacetic anhydride.
3. HPLC model 1100 and 1100 UV Detector (Agilent, Santa Clara, CA).
4. Protein C_4 (250 × 4.6 mm, 10-μm particle size) reversed-phase (RP) column (Vydac, Hesperia, CA, USA).
5. Acetonitrile.
6. Water.

2.7. Enzymatic Digestion of Acetylated Protein Sample

1. Immobilized trypsin bead slurry (Poroszyme, Applied Biosystems/Life Technologies, Carlsbad, CA).
2. Compact Reaction Column (Affymetrix/USB Biochemicals, Fremont, CA).

2.8. 2D LC-MS/MS with QSTAR Mass Spectrometer (Off-Line SCX Plus Online Reversed-Phase)

1. Vision Workstation (AB, Foster City, USA) equipped with a Polysulfoethyl A 100 × 4.6 mm, 5 μm, 300 Å strong cation exchange (SCX) column. (Poly LC, Columbia, MD).
2. All other chemicals (Sigma–Aldrich).
3. Integrated Famos autosampler, Switchos II switching pump, and UltiMate micro LC system (Dionex/LC Packings, Sunnyvale, CA).
4. Hybrid Quadrupole-TOF LC/MS/MS Mass Spectrometer (QSTAR Pulsar i) equipped with nano-electrospray ionization source (Proxeon, Odense, Denmark), fitted with 10-μm fused Silica emitter tip (New Objective, Woburn, MA).
5. 75 μm × 15 cm C18 PepMap Nano LC column (3 μm, 100 Å) (Dionex/LC Packings).
6. 300 μm × 5 mm C18 PepMap guard column (5 μm, 100 Å) (Dionex/LC Packings).

2.9. Denaturing IEF-FFE of Intact Proteins

1. BD™ FFE System with 0.4-mm spacer (Becton Dickinson, Franklin Lakes, NJ).
2. Glycerol (>99.5%), isopropanol (100%), methanol, (HPLC grade), TFA (HPLC grade), glacial acetic acid.
3. BD™ FFE-IEF kit (Becton Dickinson), pH 3–10 gradient kit, which includes IEF-free flow electrophoresis (FFE) hydroxypropyl methyl cellulose (HPMC) powder; pI mix and buffers 1, 2, and 3 for the pH 3–10 gradient.
4. Microtest flat bottom 96-well plates (Thermo Fisher Scientific).
5. 1 M sodium hydroxide: 16.0 g sodium hydroxide, Milli-Q water (Millipore, Billerica, MA) to 400.0 g.

6. 5 M sulfuric acid: 49.0 sulfuric acid, 73.3 g Milli-Q water.
7. 1 M sulfuric acid: 9.81 g sulfuric acid, 94.67 g Milli-Q water.
8. Anodic stabilization medium: 3.0 g 5 M sulfuric acid, 72.0 g urea (Serva Electrophoresis GmbH, Heidelberg, Germany), 6.8 g mannitol, 69.7 g Milli-Q water.
9. Separation medium 1: 30.3 g IEF-FFE buffer 1, 72.0 g urea, 6.8 g mannitol, 69.7 g Milli-Q water.
10. Separation medium 2: 100.0 g IEF-FFE buffer 2, 144.0 g urea, 13.6 g mannitol, 100.0 g Milli-Q water.
11. Separation medium 3: 37.0 g IEF-FFE buffer 3, 72.0 g urea, 6.8 g mannitol, 63.0 g Milli-Q water.
12. Cathodic stabilization medium: 30.0 g 1 M sodium hydroxide, 144.0 g urea, 13.6 g mannitol, 170.0 g Milli-Q water.
13. Counterflow medium: 360.0 g urea, 33.7 g mannitol, 500.0 g Milli-Q water.
14. Electrolyte anode buffer: 40.0 g 1 M sulfuric acid, 360.0 g Milli-Q water.
15. Electrolyte cathode buffer: 40.0 g 1 M sodium hydroxide, 360.0 g Milli-Q water.
16. 0.8% HPMC solution (see Note 1): 8.0 g HPMC powder, 1,000.0 g Milli-Q water. Add water to large, clean glass vessel with a large stirrer bar. Stir the water on a stirring plate until a strong vortex is created so that the base of the vortex is touching the stirrer bar. Gradually add the HPMC powder over a 10-min period. Allow mixing to occur overnight and then store at 4°C in the dark.
17. 0.1% HPMC solution: 105.0 g 0.8% HPMC solution, 525.0 g Milli-Q water.

2.10. Microscale Reverse-Phase Purification of IEF-FFE Protein Fractions

1. C4 ZipTips™ (Millipore).
2. Water, HPLC grade.
3. Acetonitrile, HPLC grade.
4. TFA, HPLC grade.

2.11. FTICR-MS

1. Apex-Qe Qq-FTICR mass spectrometer (Bruker Daltonics, Billerica, MA) equipped with a 7-, 9.4-, and 12-T actively shielded magnet and an Apollo II (Bruker Daltonics) ESI source.
2. Mass spectrometry-grade Chromasolv solvents: Acetonitrile, 0.1% formic acid and water, 0.1% formic acid.
3. HPLC-grade water, acetonitrile, and methanol.
4. Acetic acid and formic acid.
5. Amber glass with teflon tops to store solvents.

6. Ammonium acetate (Sigma).
7. Protein standards for calibration: Ubiquitin, myoglobin, and carbonic anhydrase.

2.12. Post-FFE HPLC-FTICR

1. UltiMate 3000 (Dionex, Sunnyvale, CA) HPLC pumps.
2. 200 μm ID polystyrene divinylbenzene (PS-DVB) monolithic columns (Dionex).
3. 0.05% heptafluorobutyric acid (HFBA).
4. Acetonitrile.
5. 12T Apex-Qe FT-ICR mass spectrometer.

3. Methods

3.1. Phosphorylation Analysis

A wide variety of enzymatic processes can be used to better study and understand the phosphorylation of biological molecules. Phosphatases and kinases remove and add phosphate groups, respectively. These enzymes can be utilized in such a way that both phosphorylated and non-phosphorylated forms of the peptide or protein can be produced in order to characterize them. Other reagents, such as phosphatase inhibitors, can be used in conjunction with the above-mentioned enzymes to stabilize the two forms of the target peptide or protein so that they become easier to purify and study. This inhibition step becomes increasingly important when expressing proteins in an insect or mammalian system, where phosphatase activity is quite high (35). Obtaining sufficient amounts of the phosphorylated form of a peptide or protein for MS analysis is also dependent on the concentration, activity, and efficiency of the kinases (35, 36).

Once the phosphorylated peptide or protein is stabilized and purified, comparison with its non-phosphorylated form via MS can be straightforward. The addition of a phosphoryl (HPO_3) group to a protein adds 80 Da (79.966 Da for high-accuracy measurements) to the molecular weight of the original peptide. In the resulting mass spectrum, this phosphorylated peptide shows a shift of +80 Da reflecting the added HPO_3 moiety. MS/MS can then be used to locate the site of phosphorylation on the peptide (Fig. 9).

CID MS/MS analysis performed on phosphorylated tyrosines, serines, and threonines tends to initiate the loss of negatively-charged PO_3^- (79 Da) or $H_2PO_4^-$ (97 Da) in the negative ion mode or HPO_3 (80 Da) and/or H_3PO_4 (98 Da, corresponding to HPO_3 plus H_2O) in the positive ion mode (7). H_3PO_4 is neutral in charge and, thus, is termed *neutral loss*. By performing a neutral loss scan, the phosphorylated peptides $(M+H)^+$ can be detected by searching for a fragment ion $(M+H\text{-}98)^+$ that corresponds to a loss of 98 Da from the precursor ion (37, 38). In the negative ion mode, phosphopeptides

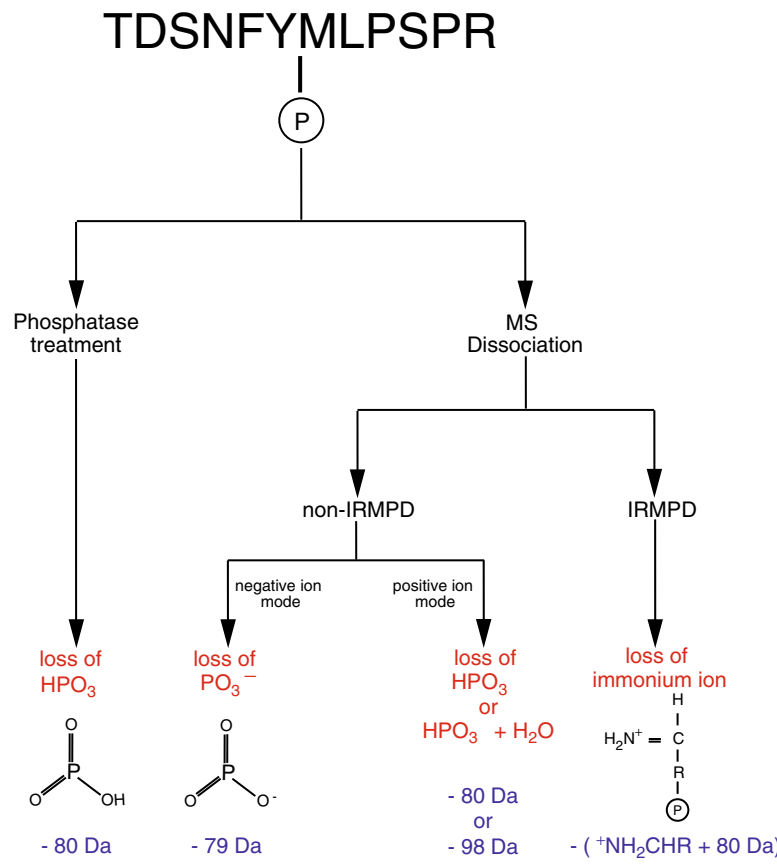


Fig. 9. Fragmentation methods of a peptide containing a phosphorylated tyrosine. Several methods, each with its characteristic losses, can be employed to study the presence and location of phosphorylations on a given peptide. Under positive ion mode of the mass spectrometer, either an HPO_3 molecule can be lost, or an HPO_3 molecule and an H_2O molecule from another amino acid can be lost ("neutral loss"). Of all the MS dissociation techniques, infrared multiphoton dissociation (IRMPD) most often generates immonium ions, which are useful for directly pinpointing the location of a PTM. Phosphatase treatments are often used to remove the phosphorylation prior to MS dissociation-based methods.

can be detected by searching for ions that generate ions at m/z 79 and/or 97 Da in the precursor ion scanning mode. Using MS/MS sequence information along with neutral loss and/or precursor ion scanning, the site of phosphorylation can often be localized.

A simple experimental study can be followed from start to finish, demonstrating the main concepts of phosphorylation analysis using MS. The p53 protein is known for its oncogenic properties in human cells; its phosphorylation state and activity were the focus of a paper by Merrick et al. (36) that serves as the example here. In brief, a wild-type form of p53 was recombinantly expressed via the baculovirus system. Okadaic acid, a phosphatase inhibitor, was used at the end stages of expression to obtain the hyperphosphorylated form. Both forms of the protein (basal and hyperphosphorylated)

were purified using immunoaffinity chromatography and were then subjected to tryptic digestion to yield proteolytic peptides. MALDI-MS analysis showed that certain peaks were shifted by 80 Da in the hyperphosphorylated sample, indicating the possible presence of phosphorylated peptides (Fig. 10). CID was used to induce fragmentation, and MS/MS was used to determine the amino acid residue to which the phosphoryl group was bound (Fig. 11) (36).

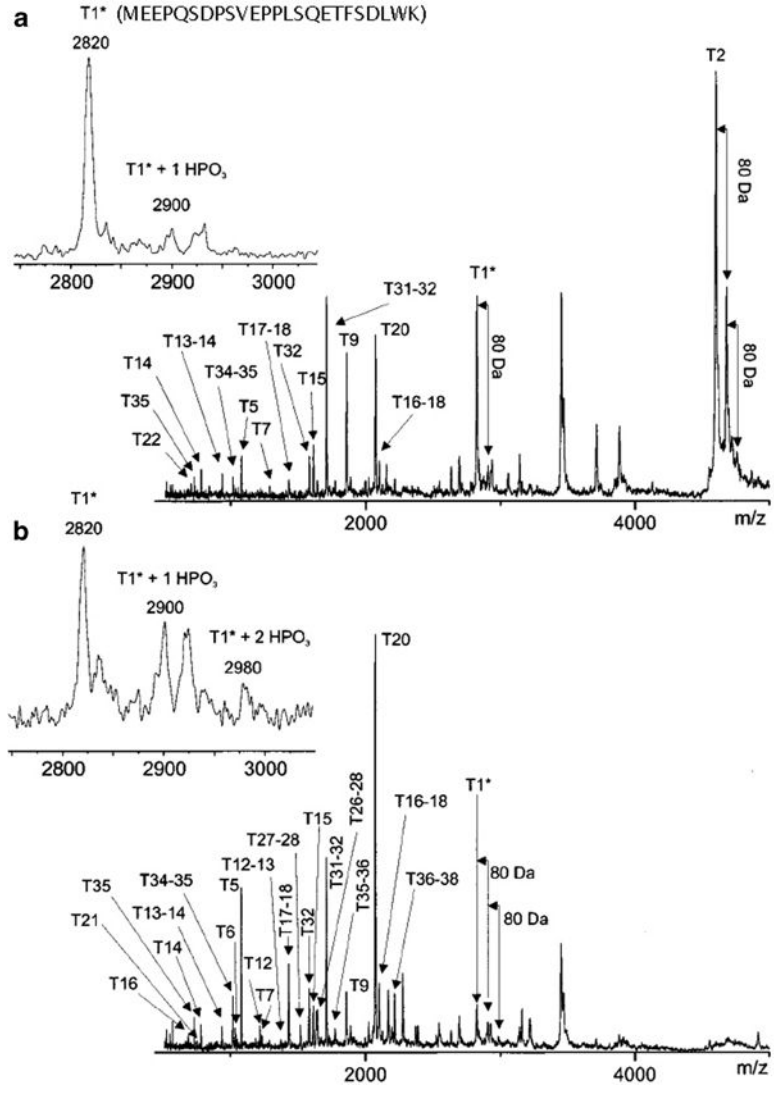


Fig. 10. MALDI-MS spectra of the tryptic digest of p-53 protein without okadaic acid (OA) treatment (**a**) and with treatment (**b**). Each tryptic fragment of the protein is denoted as Tn, where n = fragment number (from N to C-terminus on the primary sequence of the protein). The phosphorylated forms of fragments, T1 and T2, are labeled. Without treatment, the inset panel shows the dephosphorylated and monophosphorylated forms of the T1 peptide (**a**), whereas with OA treatment, the inset panel shows the dephosphorylated form as well as two phosphorylated forms (**b**). Reprinted from ref. 36, with permission.

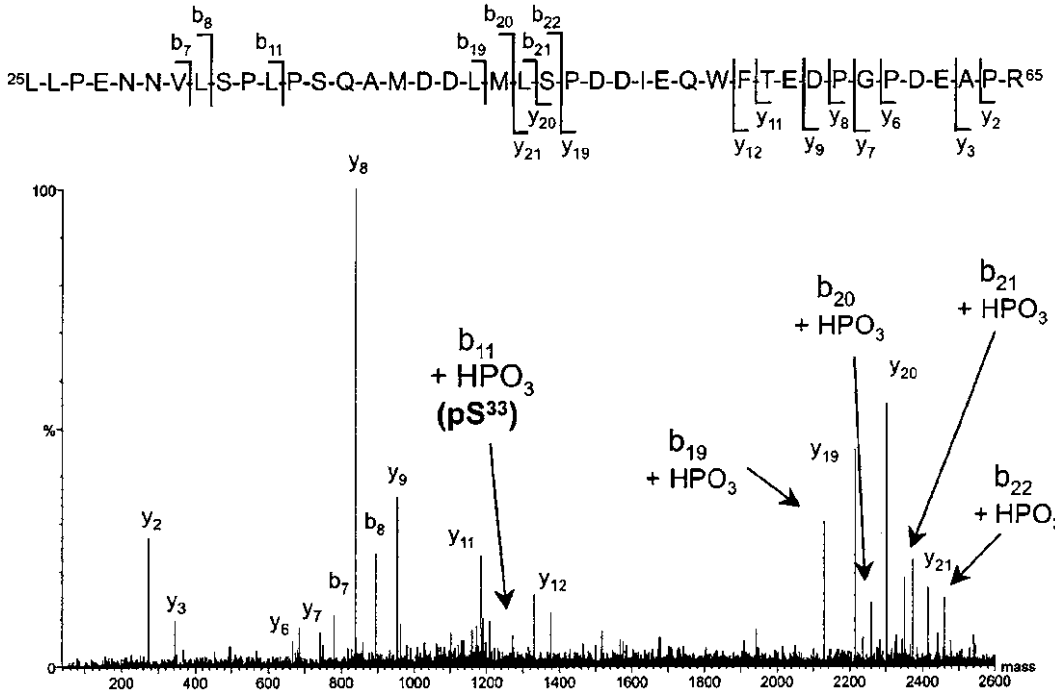


Fig. 11. Phosphorylation of Ser[33] (residue 33 on the primary sequence of the protein) determined by ESI MS/MS of the T2 peptide, shown in Fig. 10a, of p-53 protein treated with okadaic acid. Reprinted from ref. 36, with permission.

3.2. In-Solution Digestion of Proteins

1. Reconstitute Promega trypsin in 25 mM ammonium bicarbonate pH 8 to obtain a 1 µg/µL solution of trypsin. For a Glu C digest, reconstitute the Glu C to obtain a 1 µg/µL solution.
2. Dissolve sample in 20 µL of 25 mM ammonium bicarbonate solution, and add the appropriate amount of trypsin such that a 1:50 enzyme-to-substrate ratio is obtained. For a Glu C digest, 1:100 enzyme:substrate can be used.
3. Vortex and centrifuge at 6,000 rpm (2,000×*g*) in a mini-centrifuge.
4. Incubate overnight in a sealed micro-centrifuge tubes at 37°C.

3.3. MALDI-MS and MS/MS

Spotting sample and Calibrant

1. Add 6 mg of CHCA and 3.6 mg ammonium citrate into 2 mL of 50% acetonitrile: 0.1% TFA.
2. Spot 0.5–1 µL of sample onto the MALDI target plate and allow the sample to dry.
3. Apply 0.5 µL of the matrix and allow it to dry completely.
4. Create a calibration mix that contains 1:20 (v/v) of AB Sciex Mass Standards kit to matrix.
5. Spot the diluted calibration mix onto the pre-etched calibration Spotting on the target plate and allow to dry completely.

Calibration (MS reflector mode)

6. Load the plate into the mass spectrometer and create a new spot set.
7. After starting an acquisition method and a processing method, select one of the calibration spots by using the spot plate map (see Note 2).
8. Acquire spectrum and observe signal. Ensure that the signal contains the following peaks with the acceptable resolution:

des-Arg1-Bradykinin:	904.4681	>10,000
Angiotensin:	1,296.6853	>12,000
Glu1-Fibrinopeptide B:	1,570.6774	>13,000
ACTH (1–17):	2,093.0867	>12,000
ACTH (18–39):	2,465.1989	>12,000
ACTH (7–38):	3,657.9294	>10,000

Signal-to-noise ratio greater than 100 for all peaks

9. If the resolution is too low, stepwise decrease the laser setting by 100 and reevaluate the data to see if it passes specifications (see Note 3).

   Calibration and acquisition (MS/MS reflector mode)

10. Calibrate in MS/MS mode and acquire spectra according to manufacturer's protocol.
11. To search for PTMs, look for m/z of peaks that are shifted as compared to the unmodified peptides.
12. Data analysis can be performed with MASCOT software (http://www.matrixscience.com) using phosphorylation as a variable modification.

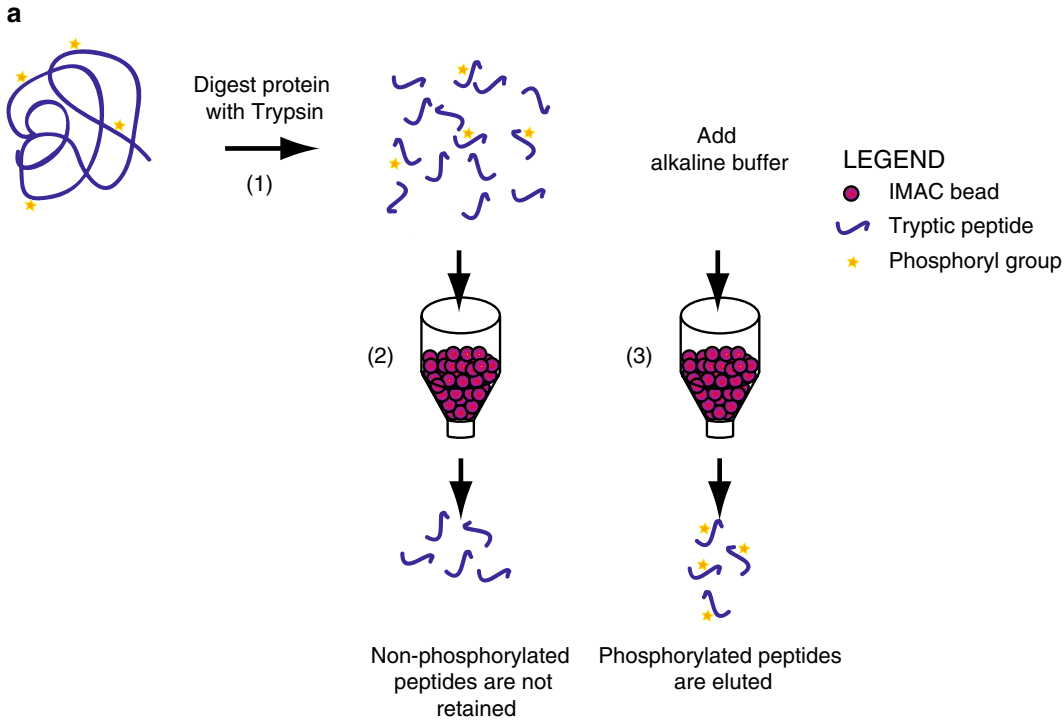
3.4. Immobilized Metal Ion Affinity Chromatography

The mass spectrometric detection of phosphopeptides is hindered in many instances by their low ionization efficiency in complex peptide mixtures (39, 40). This occurs as a result of the electronegativity of the phosphoryl group as well as the large abundance of non-phosphorylated peptides, which are also present in the sample, contributing to the previously mentioned suppression effect. Immobilized metal ion affinity chromatography (IMAC) is a technique that helps to overcome the ion suppression effect by enriching phosphopeptides from a peptide mixture (41–43). IMAC makes use of the affinity of positively-charged transition metal ions for negatively-charged phosphate groups. The metal ions most commonly used in this type of analysis include Ni^{2+}, Zn^{2+}, Ga^{3+}, Cu^{2+}, Fe^{3+}, and Ti^{4+}. In this technique, metal ions are immobilized on a chromatographic support (such as agarose beads) with chelating agents, such as triscarboxymethylethylenediamine (TED), iminodiacetate (IDA), or nitrilotriacetate (NTA). Phosphopeptides remain bound to the beads at an acidic pH and

elute at an alkaline pH. Therefore, by using an acidic solution with a pH of approximately 3–5, phosphopeptides bind to the chelated support and unbound or nonspecifically bound peptides are washed away. The bound phosphopeptides can then be eluted using an alkaline buffer (Fig. 12).

The IMAC technique is particularly suitable for use with MALDI-MS because the elution and salt removal steps necessary for ESI-MS are not needed. This minimizes potential loss of phosphopeptides. Also, IMAC beads with bound phosphopeptides can be spotted directly onto an MALDI target plate rather than pre-eluting the peptides with an alkaline buffer and spotting the eluant. In this case, the addition of the matrix solution releases the phosphopeptides from the beads (44, 45). Using IMAC in conjunction with MS allows for the accurate sequencing of phosphopeptides and determination of the specific phosphorylation site(s) (44). However, there are several disadvantages associated with the use of IMAC. One major concern is the nonspecific binding of acidic amino acid residues with the IMAC support. In order to minimize nonspecific binding of peptides, methyl esterification of the carboxyl group on acidic peptides prior to addition of the IMAC support is recommended (46). Another concern is the preferential binding of peptides with multiple phosphorylation sites to the IMAC support, leading to difficulties in the recovery of multiply-phosphorylated phosphopeptides.

1. IMAC columns are prepared by adding 30 µL of a 50% slurry of Ni-NTA resin into a compact reaction column.
2. The column is then washed with 30 µL of 100 mM EDTA three times to remove all of the unbound Ni^{2+} metal ions.
3. The column is washed sequentially with 30 µL water (three times), 30 µL 0.1 M acetic acid (three times), and 30 µL 60 mM $GaCl_3$ or 100 mM $FeCl_3$ (three times) to load the column with Ga^{+3} or Fe^{+3} metal ions.
4. The column is then washed with 30 µL water (three times), followed by 30 µL 0.1 M acetic acid (three times) to remove unbound metal ions.
5. The protein digest (2 µg protein digest dissolved in 50 µL 0.1 M acetic acid) is loaded onto the IMAC column to bind the phosphopeptides to the metal chelator.
6. The column is incubated at 37°C for 30 min on a rotating mixer.
7. The column is washed with 50 µL water (three times) and 50 µL 0.1 M acetic acid (three times) to remove less tightly bound analytes.
8. An aliquot of resin (0.5 µL) is placed directly onto the MALDI target plate followed by a 0.5-µL aliquot of the matrix solution (see Note 4).
9. The sample is allowed to dry at room temperature before loading the plate into the mass spectrometer.



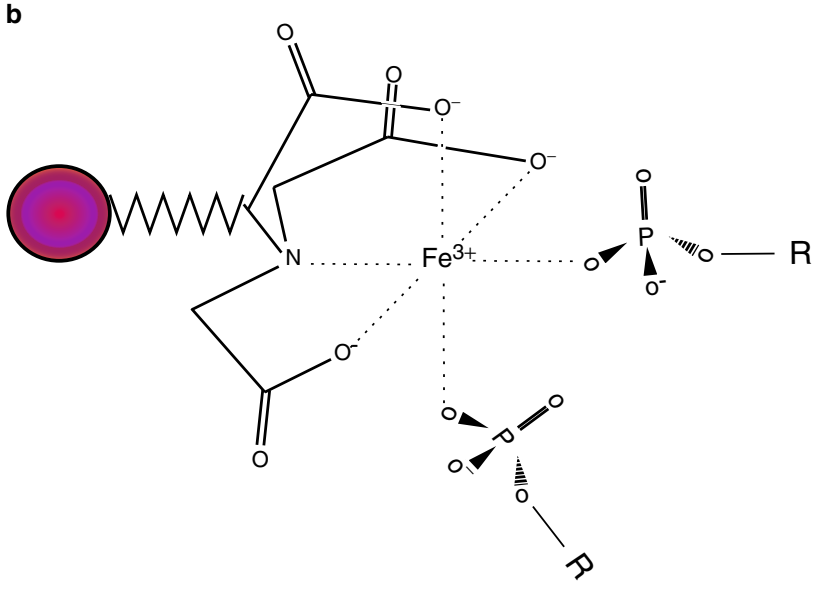


Fig. 12. IMAC. (**a**) To enrich phosphopeptides out of a complex mixture of peptides using IMAC, a phosphorylated protein is proteolytically digested. Then, the mixture of peptides is incubated with the IMAC beads and the phosphopeptides are retained while other peptides are washed off the column. A subsequent elution step removes the phosphopeptides from the column for downstream applications, such as mass spectrometry. (**b**) The surface chemistry of the bead enables the use of metal ions for affinity chromatography. These metal ions show high affinity for negatively-charged phosphoryl groups, allowing for the enrichment of phosphorylated peptides.

3.5. On-Target Dephosphorylation to Determine the Sites of Phosphorylation on Peptides

Unphosphorylated peptides are associated with higher MS sensitivities than the corresponding phosphopeptides (47, 48). As a result, the sites of phosphorylation on proteins may be examined using dephosphorylation experiments. A method called phosphatase-directed phosphorylation site determination (PPD) combines IMAC enrichment and phosphatase treatment to study phosphopeptides (Fig. 13) (27). Phosphatases are used to improve the ionization efficiencies of peptides in the positive ion mode by removing the negatively-charged phosphoryl groups. Using this technique, phosphopeptides are first attached to IMAC beads. A small volume of bead slurry (with phosphopeptides still bound) is spotted onto the MALDI target plate. The beads are then subjected to phosphatase treatment directly on the target and analyzed by MALDI-MS and MS/MS, resulting in the identification of IMAC-bound peptides including potential phosphopeptides. Examination of the sequence allows identification of the formerly phosphorylated peptides, and also allows one to determine the total number of possible phosphorylation sites (Ser, Thr, Tyr). From this data, a comprehensive mass list of phosphorylated peptides is calculated, containing every possible phosphorylation state for each peptide (27).

Hypothesis-driven-mass spectrometry (HD-MS, (49)) now comes into play. This is the application of previously-generated theoretical data (the comprehensive mass list described above) to direct the acquisition of MALDI-MS/MS data. In this case, MS/MS data is acquired on all of the calculated masses for all of the possible phosphorylated forms of the peptides that might be bound to the IMAC beads. If the suspected/hypothesized peptide is, in fact, present, the resulting MS/MS spectrum can be used to determine the specific phosphorylation site on the peptide (Fig. 14).

When using PPD with MALDI-MS, elution buffers that are incompatible with MS, such as buffers that contain ionizable salts or detergents, must be avoided. However, this technique does eliminate the need for additional cleanup steps and, because off-line chromatographic steps are minimized, this reduces the potential for sample loss (27). A benefit of using MALDI-MS (as compared to LC/ESI-MS) is that multiple MS and MS/MS analyses are obtainable from a single sample spot. This can increase the total information acquired from a single sample.

1. Acidify the sample containing the digested peptide with 100 mM acetic acid.
2. Spot 0.25–0.4 μL of a peptide solution (or peptides bound to IMAC beads) on an MALDI target plate immediately followed by the same volume of PPase diluted to various concentrations in 100 mM ammonium bicarbonate (i.e., 1:10, 1:100, and 1:500 in ammonium bicarbonate).

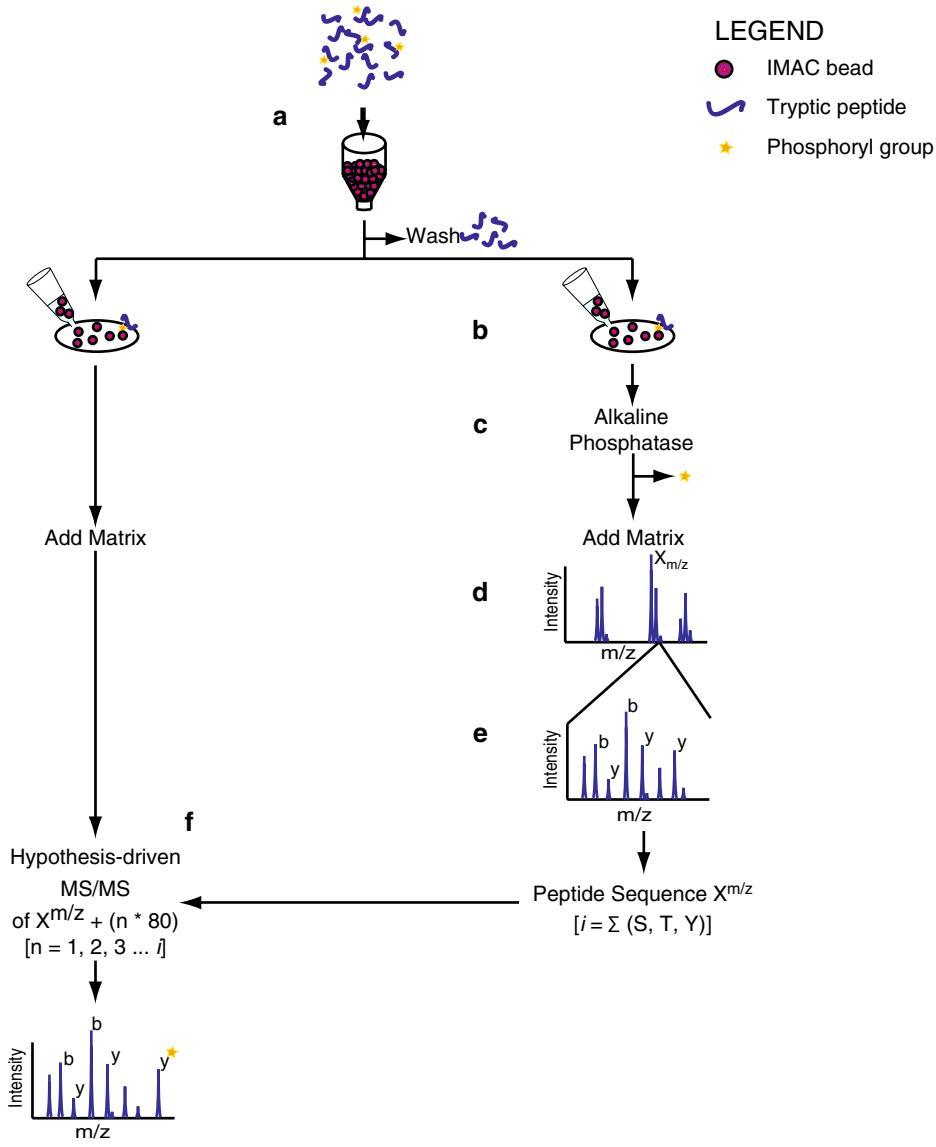


Fig. 13. PPD. Phosphopeptides from a protein digest are bound to IMAC beads and washed to eliminate nonbinding peptides (**a**). The beads are split and dispensed directly onto an MALDI target plate (**b**), where they are immediately crystallized with matrix or reacted with alkaline phosphatase (**c**). Peptides corresponding to ion signals that appear in the MALDI MS spectrum of the phosphatase-treated sample (**d**) are sequenced by MALDI MS/MS (**e**). Based on the mass of the dephosphorylated peptide ($\chi_{m/z}$) and its sequence (which provides the number of potential phosphorylation sites [*i*]), a series of hypothesis-driven MS/MS experiments are conducted on the IMAC-enriched phosphopeptides at *m*/*z* values corresponding to the phosphorylated form of each sequenced peptide for the phosphatase-treated sample (i.e., $[\chi_{m/z}+(n\times 80)]$, where $(n=1, 2, 3, \ldots i)$) (**f**). Reprinted from ref. 27, with permission.

3. Allow the phosphatase reaction to progress at room temperature for various time periods (i.e., 1 min, 2 min, etc).
4. Add 0.5–0.8 µL saturated matrix solution (see above) to the phosphatase-containing spot and allow the solution to

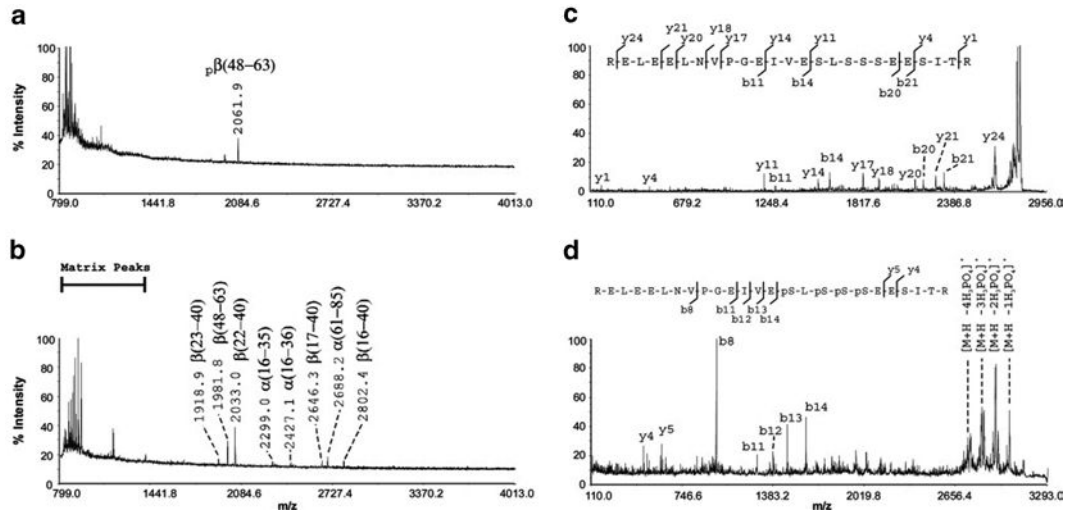


Fig. 14. (**a**) PPD of β-casein phosphopeptides immobilized on IMAC beads. MS spectrum of Fe (III)-charged IMAC beads after incubation with a β-casein tryptic digest. (**b**) Same as in (**a**), but treated with alkaline phosphatase. Peptides are annotated as follows: β-casein (β), α-S2 casein (α). Each peptide's position within the primary sequence of the protein is given in parenthesis. (**c**) MS/MS spectrum from the β (16–40) peak at *m/z* 2802.4. (**d**) HD MS/MS spectrum from the β-casein tetraphosphopeptide at *m/z* 3122.4 directly bound to IMAC beads. Reprinted from ref. 27, with permission.

crystallize at room temperature. Alternatively, CHCA in 50% acetonitrile/0.1–1% phosphoric acid can be used as the matrix solution.

5. Use 4800 MALDI-TOF/TOF for mass spectrometric analysis (see Subheading 3.3 above).

3.5.1. Phosphatase Optimization

1. Incubate proteolytically-digested sample in batch mode with IMAC beads in a compact reaction column.
2. Remove unbound peptides by washing, and spot a fraction of the phosphopeptide-containing beads directly on the MALDI target surface to be dephosphorylated. Spot another fraction of the beads which remain phosphorylated for comparison.
3. Dephosphorylate the first fraction of IMAC-bound peptides by on-target addition of PPase to the beads.
4. Add CHCA matrix to the dephosphorylated beads and then analyze by MALDI-TOF MS.
5. Analyze the peptides in the mass spectrometer using MS/MS to reveal the peptide sequence and the total number of Ser, Thr, and Tyr residues that could potentially be phosphorylated.
6. Conduct HD-MS/MS experiments at *m/z* values corresponding to each potential phosphorylation state (i.e., (M + H + 80), (M + H + 160), (M + H + 240), etc.) of the given peptide on the second aliquot of the IMAC beads that have been spotted but not dephosphorylated.

3.6. Acetylation Analysis

As mentioned previously, MS/MS dissociation can yield internally fragmented immonium ions. These ions are useful in acetylation analysis. For the determination of the acetylation site on a peptide, MS/MS data can be generated using precursor ion scanning or LC-MS/MS experiments and searched for an immonium ion with an m/z that is characteristic of acetylation (50). An immonium ion of m/z 199 is indicative of an acetylarginine residue (29). Similarly, the fragmentation of acetylated lysine yields an immonium ion of m/z 143.1 (29, 50). However, this ion is not exclusive to acetylated lysine and could also result from internal fragment ions of other peptides as well. A more reliable characteristic fragment ion for acetyllysine is seen at m/z 126.1, which is the immonium ion, with a secondary loss of ammonia (50) (Fig. 15).

Not only can naturally acetylated peptides be analyzed, but unmodified proteins can be artificially acetylated prior to proteolytic digestion. This artificial acetylation is done to locate accessible residues, where these modifications occur, and, by doing this, to determine structural information on the amino groups in proteins. A low molar excess of acetic anhydride followed by high molar excess of hexadeuterated acetic anhydride can be used to

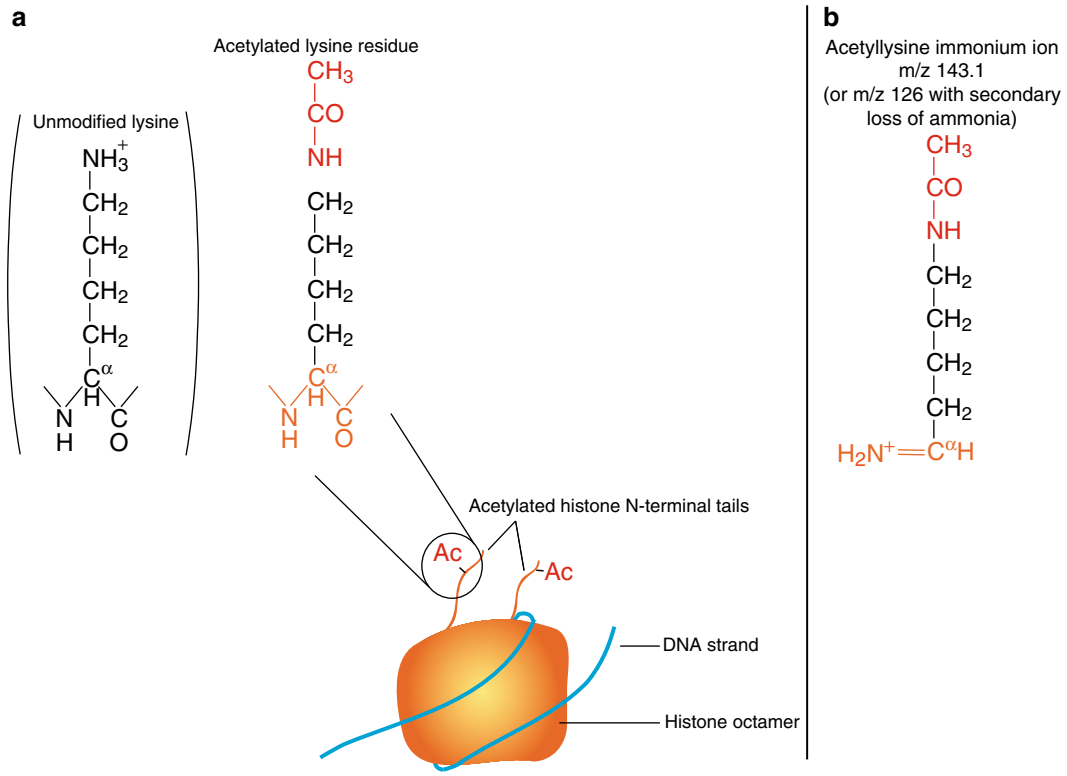


Fig. 15. Histone acetylation. (**a**) Histone acetylation is catalyzed by enzymes, histone acetyltransferases (HATs), that acetylate lysine residues on the N-terminal tails of histones H3 and H4 (part of the core histones). The formation of ε-N-acetyl lysine neutralizes lysine's positive charge and impacts downstream gene regulation. (**b**) Structure of the immonium ion of acetyllysine.

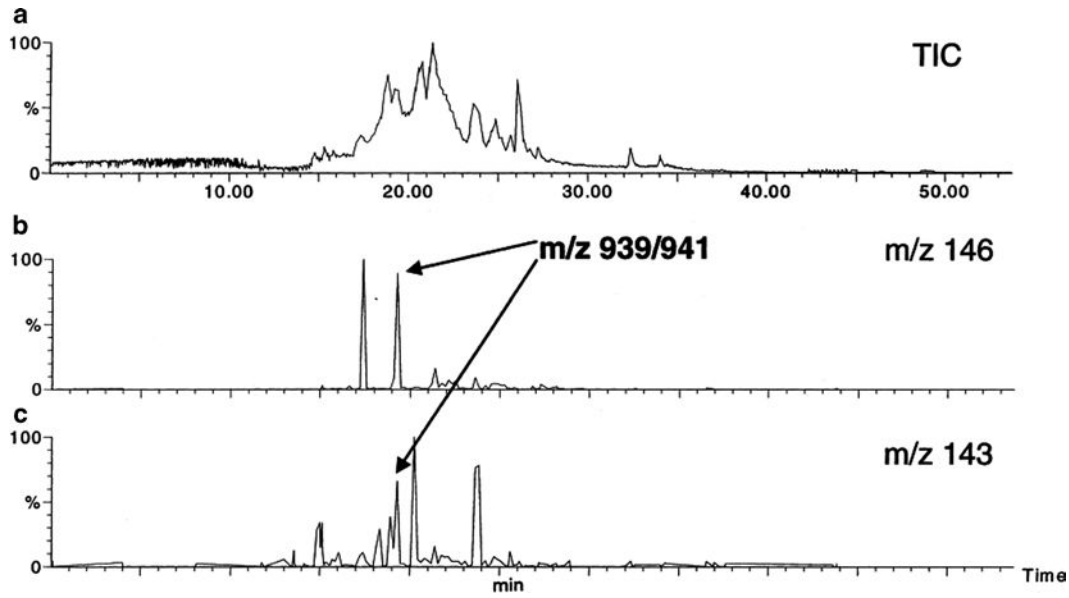


Fig. 16. Example of LC-MS/MS. LC-MS/MS of a tryptic digest of acetylated HIV-p24 protein, showing the total ion chromatogram (**a**), the extracted ion chromatogram (**b**) for the immonium ion of trideuteroacetyllysine (*m/z* 146), and the extracted ion chromatogram for the immonium ion of acetyllysine (*m/z* 143) (**c**). The ions at *m/z* 939/941 are due to the presence of [$^1H_3$]acetyllysine (acetyllysine-d_0) and [$^2H_3$]acetyllysine (acetyllysine-d_3) from a tryptic digest of HIV-p24 protein. Reprinted from ref. 29, with permission.

acetylate the protein. The ratio of the ion signals from acetylated to deuteroacetylated peptides reflects the relative reactivities of amino groups which directly correlate with their surface accessibilities (Fig. 16) (51).

3.7. Acetylation of Lysine ε-Amino and N-Terminal Amino Acid Groups

1. Add acetic anhydride in a 10–1,000-fold molar excess per amino group (added to 0.5 mL of protein solution (10 μg/μL in 200 mM NH_4HCO_3) (see Note 5)).
2. Carry out the reaction for 30 min at 22°C. The pH during the hydrolysis of the anhydride is maintained at 6.5 by addition of 25% NH_3.
3. Desalt the acetylated peptides, and exchange the buffer for a methanol–water mixture (50:50, v/v) by solid-phase extraction using a C18 Sep-Pak cartridge following the manufacturer's instructions (52) as described in steps 4–7 below.
4. Condition the cartridge using an organic solvent (methanol or acetonitrile, depending on what the sample is dissolved in) followed by a weak solvent to wet the stationary phase.
5. Load the sample onto the cartridge.
6. Wash the sample with water.
7. Elute the retained acetylated peptides using a solution with a solvent containing a higher organic concentration, 50:50 methanol:water or 50:50 acetonitrile:water (see step 4 above).

3.8. Two-Step Acetyation/Tri-Deuteroacetylation of Sample Protein

1. Dilute sample with 200 mM NH_4HCO_3 to a final concentration of 33 ng/μL.
2. Use a 10,000-fold molar excess of acetic anhydride for partial modification using the reaction conditions described above.
3. Add a 100,000-fold molar excess of hexadeuteroacetic anhydride to obtain complete modification.
4. The acetylated sample can now be purified by HPLC using a binary pumping system (for example, an Agilent model 1100) and an Agilent model 1100 UV detector. For this purification, use the following conditions:

   Column: Protein C_4 (250×4.6 mm, 10-μm particle size) reversed-phase column (Vydac, Hesperia, CA, USA).

   Solvents: 90% acetonitrile and water (both should contain 0.1% TFA).

   Flow rate: 1 mL/min.

3.9. Enzymatic Digestion of Acetylated Protein Sample

1. Dissolve the HPLC-purified protein (approximately 100 μg) in 20 μL of acetonitrile-deionized water (50:50, v/v).
2. Reduce the acetonitrile concentration to 10% by adding 80 μL of 50 mM NH_4HCO_3 to yield a final volume of 100 μL.
3. For proteolytic digestion, add 15 μL of immobilized trypsin bead slurry and incubate for 4 h at 37°C.
4. Remove the end plug from the compact reaction column containing the IMAC beads (Subheading 3.4), and push the sample digest through the IMAC column into a micro-centrifuge tube.
5. Perform the LC-MS/MS experiments as outlined below.

3.10. 2D LC-MS/MS with QSTAR Mass Spectrometer (Off-Line SCX Followed by Online LC/ESI-MS/MS)

3.10.1. First Dimension: Strong Cation-Exchange HPLC

1. Put the polysulfoethyl column onto the off-line HPLC system.
2. Prepare buffer A: 10 mM KPO_4 (pH 2.7), 25% acetonitrile.
3. Prepare buffer B: 10 mM KH_2PO_4, 25% acetonitrile, and 0.5 M KCL.
4. Set flow rate to 0.5 mL/min.
5. Bring samples up to 2 mL with buffer A and inject onto column.
6. Allow column to equilibrate for 20 min in buffer A before a gradient is applied; 0–35% B in 30 min.
7. Collect fractions every min after injection.
8. Reduce the volume of collected fractions in a Speed-Vac and transfer to autosampler vials.

3.10.2. Reversed-Phase HPLC Coupled Online to QSTAR for MS Analysis

1. The mobile phase (solvent A) consists of water/acetonitrile (98:2, v/v) with 0.05% formic acid for sample injection and equilibration on the guard column at a flow rate of 100 μL/min.

A linear gradient will be created – after switching the trapping column inline – by adding solvent B, which consists of acetonitrile/water (98:2, v/v) with 0.05% formic acid.

2. Reduce the flow rate to 200 nL/min for high-resolution chromatography and introduce into mass spectrometer.
3. Set curtain gas for the mass spectrometer at 23, use nitrogen as the collision gas, and use 2,700 V as the ionization tip voltage.
4. Bring samples up to a volume of 20 μL with 5% acetonitrile and 3% TFA. Transfer to autosampler vials.
5. Inject 10 μL of sample in 95% solvent A and allow to equilibrate on the trapping column for 10 min to wash away any contaminants.
6. Switch the trapping column in-line with the MS, and start a 40-min linear gradient from 5% B to 60% B over 40 min. Over the following 5 min, increase the composition of the mobile phase linearly from 5 to 80% B before decreasing the concentration to 5% B at 5.1 min. Reequilibrate the column for 15 min before the next sample injection.
7. Acquire MS data automatically using Analyst 1.0 software.
8. Use an information-dependent acquisition method consisting of a 1-s TOF MS survey scan over the mass range m/z 400–1,200 and two 2.5-s product ion scans of mass range m/z 100–1,500.
9. Select the two most intense peaks that have at least 20 counts, with charge state 2–5, for fragmentation.
10. Use a 6 Da window to prevent those peaks from the same isotopic cluster from being fragmented again.
11. Once an ion has been selected for MS/MS fragmentation, put it into an exclude list for 180 s.

Ubiquitination Analysis

Ubiquitination plays an important role in a number of cellular processes, such as degradation and transcriptional regulation of histones (53–57). Ubiquitination is characterized by the attachment of the C-terminus of the ubiquitin protein to the ε-amino group of an internal lysine residue of the target protein, forming a covalent bond (58). Additional ubiquitin moities are added to the initial ubiquitin, forming polyubiquitin chains. MS analysis of ubiquitination is facilitated by protolytic digestion of this modified protein (59, 60). For example, in the case of ubiquitination of histone H2A, the sequence at the C-terminus of the ubiquitin molecule is … RLRGG; in this case, the last "G", glycine, is covalently linked to a lysine residue on H3. Subsequent trypsin digestion not only cleaves at the C-termini of all lysine and arginine residues in H2A, but also cleaves at the C-terminus of the arginine in "LRGG" on

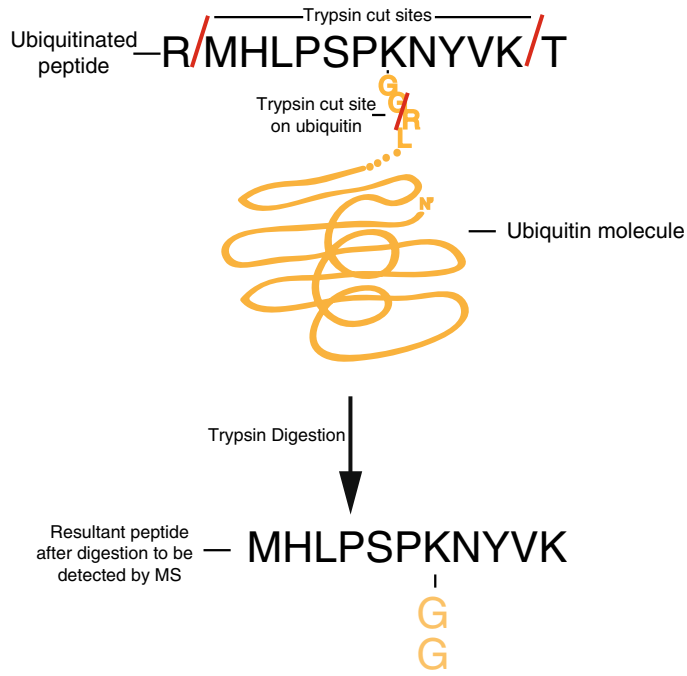


Fig. 17. Ubiquitination of proteins. The process of ubiquitination involves the attachment of the C-terminus of ubiquitin to a lysine in the peptide. Trypsin digestion results in the cleavage of the ubiquitin protein such that a glycine–glycine (GG) remains attached to the lysine. MS analysis indicates a larger *m*/*z* for peptide fragments containing a ubiquitinated site than those that do not.

the ubiquitin. Consequently, this process leaves the "GG" adduct still attached to the lysine residue on the H2A peptide (60) (Fig. 17). However, steric hindrance at the site of ubiquitin attachment can cause a missed cleavage, resulting in the adduct "LRGG" being left on H2A after cleavage (61) (Fig. 18). Alternatively, GluC endopeptidase digestion leaves a much larger portion attached to H2A, "STLHLVLRLRGG" (59) (Fig. 19). The presence of these additional residues on the target peptide allows for MS probing of ubiquitination sites.

Once MS/MS data has been acquired and analyzed, the peptide and its ubiquitination site can be identified by database searching. Each MS/MS spectrum can be searched against a protein database to find potential modified peptides. The search can also be done by specifying the elemental composition or the molecular mass of the adduct as a variable modification. For instance, if the sample was digested with trypsin, 114.1 Da (Glycine–Glycine) can be entered as a variable modification. This approach has been successfully used in the past to find ubiquitination sites (60). However, low-abundance peptides are difficult to detect with this search method (62). An alternative option is to use software to create a mass list of all possible peptides from the protein and their hypothetical masses if they were ubiquitinated. The mass list is used to

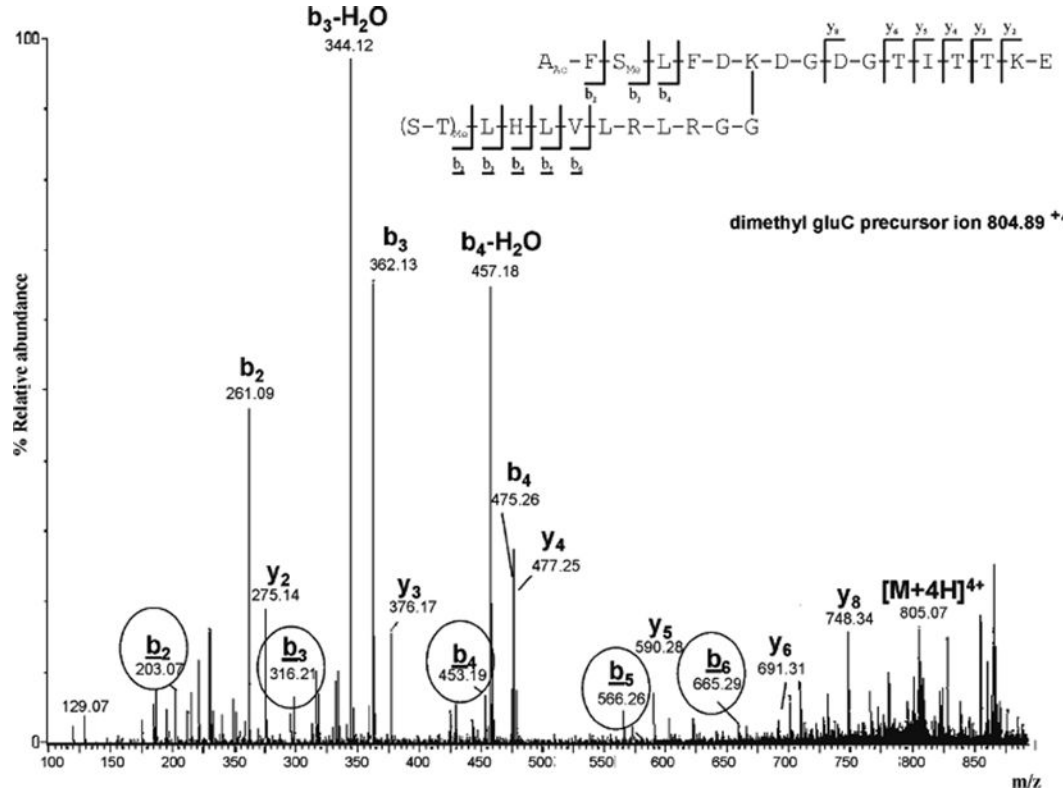


Fig. 18. MS/MS spectrum and fragment ion analysis of the model ubiquitinated calmodulin peptide (GluC fragment). The spectrum of the dimethylated form of the peptide is shown. The fragment ions b_2 to b_6 of the ubiquitin residual peptide attached to calmodulin are circled on the spectrum. These ions can be used as signature ions for ubiquitintated peptides facilitating the detection of those peptides. Reprinted from ref. 61, with permission.

search the MS data for any possible modified peptides. A different type of MS scan is then performed, where the modification-containing peptide is specified as the precursor ion and MS/MS is *only* performed on these precursor ions. This type of MS scanning, termed *include only* MS/MS by some software manufacturers, proves to be up to 100-fold more sensitive for low-abundance peptides (62). Thus far, very few research groups have successfully identified ubiquitination sites via MS (63–66). This can be attributed, in part, to the difficulty in purifying ubiquitin-bound proteins of interest due to the transient nature of the bond and low occurrence in the cell; however, studies of ubiquitinated model proteins demonstrate these concepts (61, 62).

3.11. FTICR-MS

While bottom-up proteomic techniques are most commonly used for the identification of PTMs (67), advances in dissociation techniques and FTICR-MS technology have made top-down proteomics a practical alternative for PTM characterization (68–71). The high resolution and mass accuracy of the FTICR-MS can be exploited

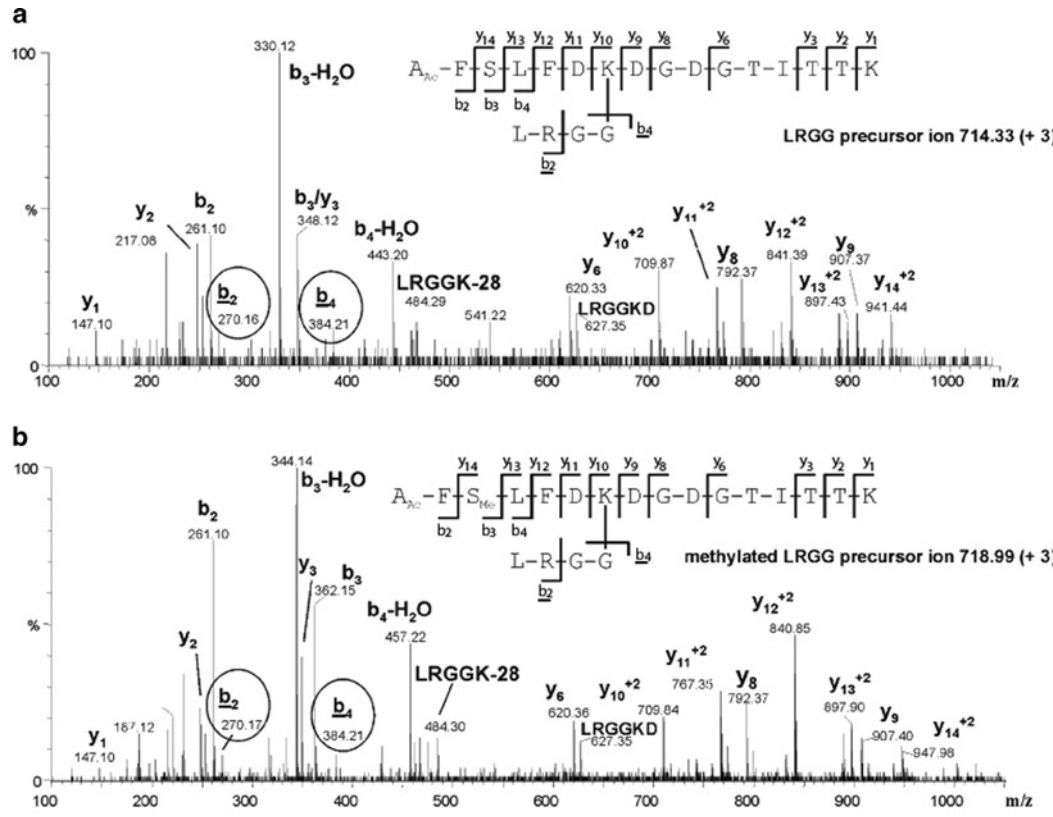


Fig. 19. MS/MS spectra of peptides obtained by tryptic digestion containing the residual ubiquitin "LRGG-tail." Fragment ion analysis of the non-methylated LRGG tryptic peptide precursor reveals ubiquitin-specific signature b ions (b_2 and b_4) as well as internal fragments (LRGGKD) (**a**). MS/MS of the methylated LRGG tryptic peptide confirms the assignment of b_2, b_4, and internal fragments ions. Reprinted from ref. 61, with permission.

to characterize the PTMs present on histones using top-down proteomic approaches. Top-down sequencing using FTICR-MS is capable of covering the entire sequence; therefore, it is especially useful where the PTMs on large peptides or proteins interfere with enzymatic cleavage sites (72). In addition, using the top-down proteomics approach, the abundance of protein isoforms may be determined directly because intact proteins are not as susceptible to instrumental biases as their smaller peptide counterparts (73). These features, combined with the ability of FTICR-MS to conduct sequential MS fragmentations (MS^n) thereby providing accurate sequence information, make FTICR-MS ideal for the identification of specific PTM sites of histones (72). The stoichiometry of the PTMs may be calculated by comparing the *m/z* values of the posttranslationally modified protein isoform with the non-modified protein isoform. To determine the exact locations of the PTMs, the protein can be fragmented within the FTICR-MS using CID or in-source fragmentation in conjunction with ECD or ETD (*see* Subheading 1.2). Furthermore, ECD and ETD have the

added advantage of being able to detect labile modifications that may not be detectable using the bottom-up approaches (23, 25). These spectra can then be analyzed either by database searching or *de novo* sequencing (i.e., manual sequencing of a molecule based on the mass differences between the fragment ions without prior knowledge of the amino acid sequence). The spectra can be automatically or manually interpreted to look for mass differences indicative of histone PTMs: phosphorylation, acetylation, methylation, and/or ubiquitination (74).

3.11.1. Preparation of Proteins for Direct Infusion

1. Commercially available proteins can be used without further processing by diluting them with a nano-ESI compatible solvent to a concentration that yields a moderately low, but detectable, signal (see Subheading 3.11.2).
2. Complex mixtures of proteins need to be separated beforehand by reversed-phase HPLC, nickel column, gel filtration, FFE, or some other analytical separation technique.
3. HPLC fractions can be sprayed directly onto the mass spectrometer after lyophilization and resuspension with spraying solvent (see Subheading 3.11.2).
4. Salts and detergents contained in fractions need to be removed with C4 ZipTips™, following the Millipore's published protocol (75). For the wetting solution, we use 100% ACN, for conditioning the column we use is 50% ACN, to wash we use 0.1% TFA, and to elute we use 50% ACN, 0.1% TFA.

3.11.2. Spraying Solvents

Prepare fresh spraying solvent. A variety of solvents can be used for top-down experiments. Solvent selection usually depends on the protein and on the experiment to be performed: for example, denatured versus native.

1. For denaturing conditions, methanol or acetonitrile work well with formic acid or acetic acid. The concentration of organic solvent should be adjusted to 50–80%, and the concentrations of the acids vary from 0.05 to 2%. It is extremely important to keep the solvents clean and to avoid glass containers that may release sodium. Sodium forms adduct ions with proteins and may complicate the spectra, making analysis and interpretation more difficult. Low-retention micro-centrifuge tubes should be used to avoid sample loss.
2. For native conditions, a 10 mM ammonium acetate solution is appropriate. If necessary, a volatile organic acid (such as formic acid or acetic acid) can be added to aid the ionization.

3.11.3. ESI Source

The Bruker Apex-Qe Qq-FTICR mass spectrometer can be equipped either with a capillary-flow (2 μL/min) or a nanospray (200 nL/min) ESI source.

1. In capillary-flow mode, the analyte is loaded into the source of the mass spectrometer using either an automated LC pump setup or syringe pump.
2. In nanospray mode, a background spray is established usually with the same solvent used to make the sample solution. And the analyte is delivered through a sample loop with the aid of a six-port switching valve (see Note 7).

3.11.4. MS Experiments

1. Turn on the instrument and set up appropriate ESI and other operation parameters. The spray voltage for nanospray ionization is 800–1200 V, and roughly 3600–4000 V for capillary-flow spray.
2. Load a standard protein, such as ubiquitin, at 0.5 μM.
3. Tune the instrument to obtain the best signal, using a data acquisition size of 512 or 1024 kilobytes per second and with broadband detection. Important tuning parameters include the background gas pressure in the collision-cell hexapole (~1.5 × 10–3 mbar), the skimmer voltage, the hexapole bias voltage, the vertical and horizontal ion beam steering voltages, and a combination of parameters applied to both the sidekick lens and the ICR cell in order for efficient ion trapping, excitation, and detection. The time of ion accumulation in the collision-cell hexapole and the time of flight (TOF) for ions to move between the two trap plates are very important and need to be carefully adjusted. Usually, a longer TOF favors trapping higher m/z ions, while a shorter TOF favors trapping low m/z ions. In addition, the ICR cell can be filled with N iterations of precursor ions which are isolated by the mass-selective quadrupole and accumulated in the collision cell during ion injection. This experimental design help to reduce the ion dwell time inside the 'high-pressure' collision cell, which could lead to unexpected fragmentation due to ion-background gas collision, etc. Usually, the ICR cell ion injection is repeated one to several times before ion excitation and detection, depending on the specific experiment.
4. Once a stable signal with high intensity is obtained, a spectrum from the standard protein is acquired and used to calibrate the instrument using the monoisotopic or other isotopic m/z values from at least three ion clusters, but with different charge states.
5. Acquire mass spectra from the sample using with the same operating parameters.
6. It is critical to keep the same parameters when acquiring the calibrant spectrum and the sample spectrum. The concentrations of the two also need to be similar because the number of ions in the ICR cell affects the calibration.

3.11.5. MS/MS Experiments

Collision-Induced Dissociation

1. The 12-T Apex-Qe Qq-FTICR mass spectrometer is equipped with a mass-selective quadrupole Q that allows isolation of the ion of interest, and a collision cell q (hexapole) that allows CID with argon as the collision gas at a pressure of 1.5×10^{-3} mbar.
2. The charge state of interest is selected by quadrupole Q_1 with a window width of 10 m/z or less. The time in the hexapole (i.e., the accumulation time) can usually be increased if fewer ions are entering the ICR cell (for example, if only a single charge state of the analyte is allowed to enter ICR cell).
3. The bias voltage applied to the hexapole as the collision energy is increased in a stepwise manner until significant fragment ions appear.
4. A CID mass spectrum is acquired. If the protein is large, the signals accumulated from multiple scans (e.g., 50–100) per spectrum are averaged in order to increase the signal-to-noise ratios of the fragments.

Electron-Capture Dissociation

1. The 12-T Apex-Qe Qq-FTICR mass spectrometer is equipped with a hollow dispenser cathode which emit electrons into the ICR cell.
2. Turn on the cathode, and increase the heating voltages *slowly* and cautiously to avoid damage (for example, use a rate of 0.1 A/min from 0 to 1.7 A).
3. Adjust the bias voltage (1–2.5 V), the electron pulse length (5–25 ms), and the lens voltages (10 to 25 V) for ECD, until fragments are observed while the parent ion intensity decreases.
4. Acquire the spectrum from an accumulation of 50 to even more than 1000 scans so as to increase the signal-to-noise ratios of the ECD fragments.

Infrared Multiphoton Dissociation

1. The 12-T Apex-Qe Qq-FTICR mass spectrometer is equipped with a BaF_2 window and a 25 W (CW) CO_2 IR laser.
2. Isolate the precursor ion of interest using the Q region.
3. Set the laser power to 35% and the irradiation time to 0.1 s.
4. Increase the irradiation time in increments of 0.1 s until fragmentation is observed.
5. Acquire the spectrum (averaging/summing 50–100 spectra).

3.11.6. CID Combined with Skimmer-Induced Fragmentation

1. Obtain fragments of all of the ions in the source ions by skimmer-induced fragmentation: the skimmer voltage is increased to indiscriminately fragment ions.
2. Mass select a fragment ion in the Q region and perform a CID experiment as previously described (see Subheading "Collision-Induced Dissociation").

Data Analysis

1. Open data file in Data Analysis software (Bruker Daltonics; version 3.4, build 179).
2. Determine the monoisotopic masses with the peak-picking Sophisticated Numerical Annotation Procedure 2 (SNAP2) module. The Bruker SNAP2 algorithm automatically determines the charge states of the experimentally-observed isotopic profiles from any singly- or multiply-charged ion cluster in a mass spectrum, accurately extracts the monoisotopic masses even for very weak multiply-charged ions, and simulates the mass spectrum for each protein or multiply-charged ion with different charge states.
3. If a graphic deconvoluted mass spectrum is desired, one can deconvolute the snapped spectrum. The molecular mass of the protein can then be readily determined.
4. To assign fragments, the data is exported after SNAP2 mass list determination to Biotools (Bruker Daltonics), which matches theoretical fragments (based on the target protein sequence) to the observed fragments.

3.12. FFE Combined with FTICR-MS

The high pI of histones may pose complications to separation using standard analysis techniques (i.e., 2D gel electrophoresis, ion-exchange chromatography) (76, 77). FFE is a suitable alternative to standard techniques because it allows liquid phase separations based on isoelectric points as well as fraction collection (Fig. 20) (78). Whereas gel chromatofocusing may be associated with protein loss due to adsorption to the gel, FFE is not (79). FFE does, however, require a cleanup step because it is necessary to remove interfering substances found in FFE buffers (e.g., salts, surfactants, ampholytes, etc.) (Fig. 21). It should be noted that the prompt removal of urea is important – removing urea can significantly reduce protein carbamylation which can be caused by the interaction of lysines with isocyanic acid, a degradation product of urea. For the same reason, samples containing urea should also be kept cold (0–4°C). For cleanup, either reversed-phase ZipTips™ or other RP separation techniques may be used. FFE is particularly well-suited for use with FTICR-MS for top-down proteomics because high-resolution separation is attainable while allowing proteins to remain in solution (79). FFE is compatible with both denaturing and nondenaturing conditions which allows for the separation of functional, intact proteins and protein complexes (Fig. 22).

3.12.1. Protein Separation via FFE

1. Ensure that assembly of the FFE system (with 0.4-mm spacer) is free of air bubbles and in compliance with the manufacturer's specifications.
2. Insert each of the FFE inlet and counterflow inlet lines into the 0.1% HPMC solution and set the media pump flow rate to 150 mL/h. Allow this to flow for 5 min (see Note 8).

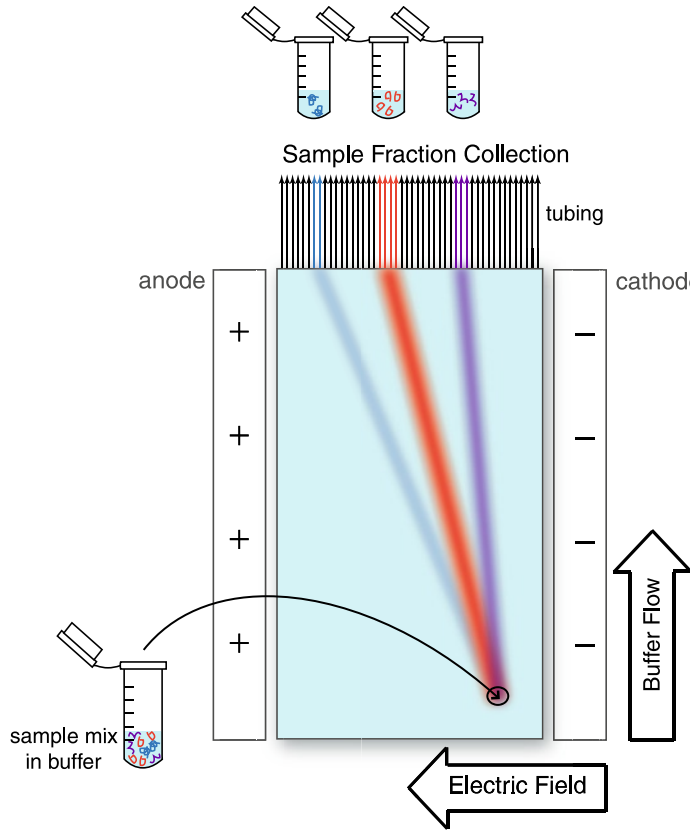


Fig. 20. Setup of the free flow electrophoresis system. The protein-containing sample mixture is introduced into the running medium through the sample inlet (*encircled*). A voltage is applied across the system allowing for isoelectric focusing in the *horizontal plane*. As the proteins are separated, the proteins travel through the chamber driven by laminar flow and are fractionated into a 96-well plate.

3. Stop the pump, remove the FFE inlet, and counterflow inlet lines from the HPMC solution and place in a 1-L beaker of Milli-Q water. Turn the pump on at 150 mL/h and allow this to flow for 5 min.
4. Stop the pump, remove the FFE inlet, and counterflow inlet lines from the water and place into the counterflow media. Turn the pump on at 150 mL/h and allow this to flow for 15 min.
5. Stop the pump and place each FFE inlet and counterflow inlet line into the following IEF buffers (see Subheading 2.9):

   Inlet 1: Anodic stabilization media

   Inlet 2: Separation media 1

   Inlet 3–4: Separation media 2

   Inlet 5: Separation media 3

   Inlet 6–7: Cathodic stabilization media

   Counterflow inlets: Counterflow media

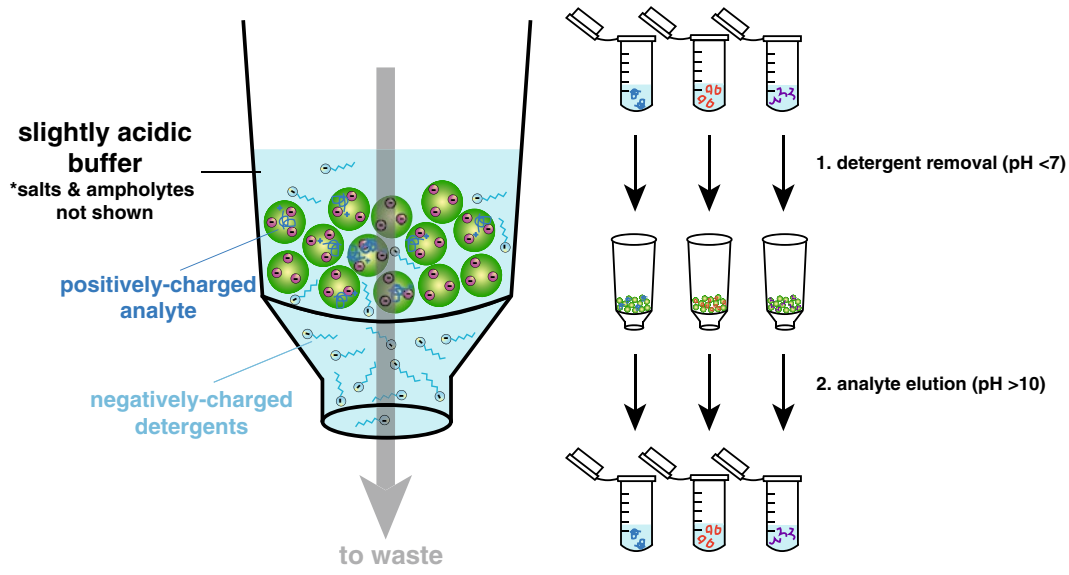


Fig. 21. FFE cleanup to remove detergents from sample fractions. Some detergents (i.e., SDS) are not suitable for use in MS. They can be removed prior to MS analysis in a number of ways. Shown here is strong cation exchange (SCX) chromatography. (1) When the analyte of interest is in a slightly acidic environment, the protein becomes protonated and carries a positive charge. In this example, negatively charged detergents are repelled by the negatively charged packing material. The positively charged protein is retained on the negatively charged packing material. (2) The packing material is then washed with a more basic solution (pH > 10) to decrease the protonation of the analyte, thereby reducing its affinity for the negatively charged packing material. In this way, the protein is eluted and collected.

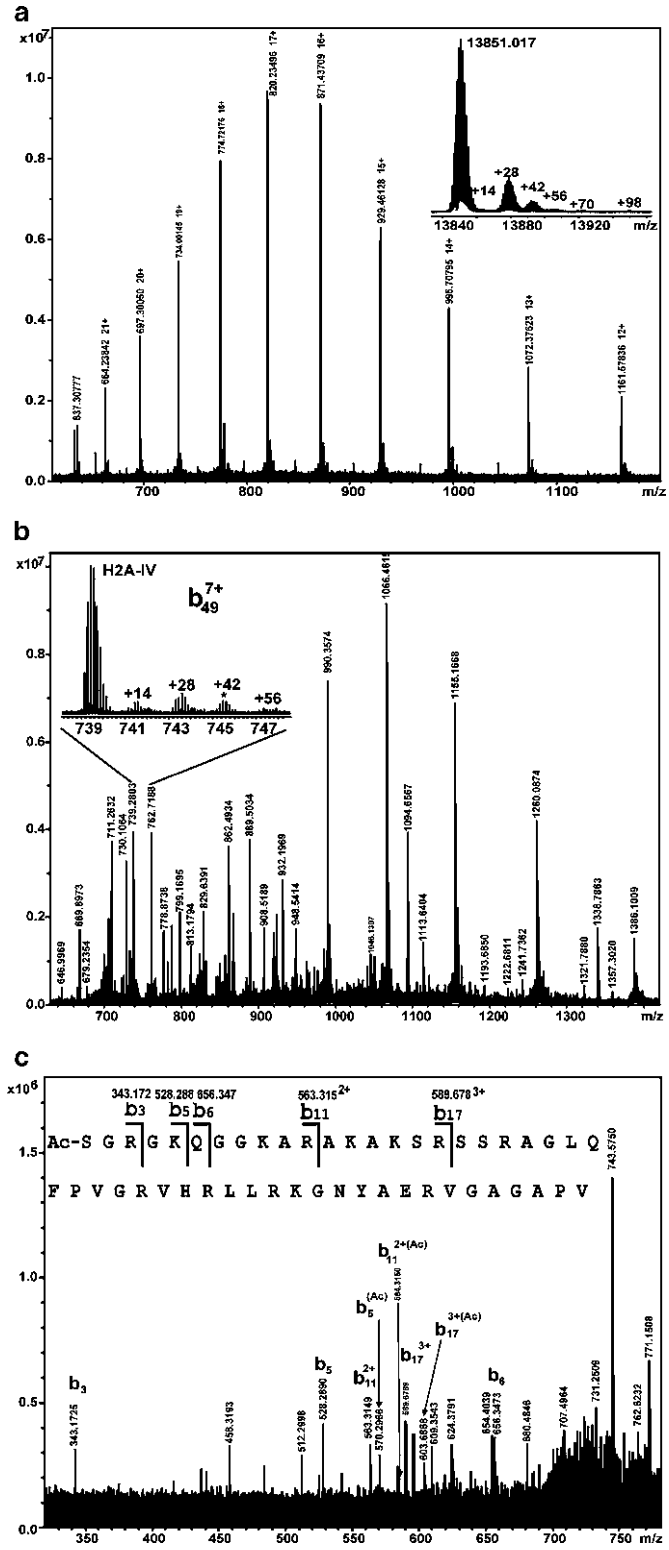
6. Run the media pump at 72 mL/h for 10 min (keep the flow going from this point on).
7. Prime the sample inlet by uncapping the inlet and allowing flow from the separation chamber for 2 min. Connect the sample inlet to the sample pump.
8. Place the electrode contact lid onto the separation chamber.
9. Set the voltage, current, and power maxima to the following:

   Voltage (V_{max}) = 650 V

   Current (I_{max}) = 50 mA

   Power (P_{max}) = 60 W
10. Turn the voltage on and allow the current to stabilize for 20 min. Once stabilized, the current should be 21 mA ± 3 mA.

Fig. 22. Identification and characterization of chicken H2AIV isoforms by top-down analysis using 12T FTMS after FFE separation. (**a**) Direct-infusion ESI-MS of a chicken blood erythrocyte histone FFE fraction (78th fraction) after C4-ZipTip desalting. This fraction was enriched in chicken H2A-IV and its various modified forms. The inset shows the deconvoluted H2A-IV average mass 13,851.017 Da (calculated mass 13,851.007 Da) corresponding to N-terminal acetylation after methionine removal. (**b**) In-source skimmer-induced MS/MS of H2A-IV isoform. The inset displays the $b49^{7+}$ ions of H2A-IVand its major modified forms. (**c**) MS^3 analysis by combined in-source and collision-induced fragmentation of $b49^{7+}$ ion (*m*/*z* 745.4) of the 142.01-Da H2A-IV isoform isolated by the quadrupole mass filter. The observed N-terminal b-series ions identified lysine-5 residue to be one of the major acetylation sites, in addition to the N-terminal acetylation.


a
13851.017
+14
+28
+42
+56
+70
+98
13840
13880
13920
m/z
637.30777
664.23842 21+
697.30050 20+
734.99146 19+
774.72175 18+
820.23495 17+
871.43709 16+
929.46128 15+
985.70795 14+
1072.37523 13+
1161.67836 12+
b
H2A-IV
b49 7+
+14
+28
+42
+56
739
741
743
745
747
990.3574
1066.4815
1155.1668
1260.0674
1336.7883
1386.1009
m/z
c
343.172 528.288 656.347
563.315 2+
588.678 3+
b3
b5 b6
b11
b17
Ac-S G R G K Q G G K A R A K A K S R S S R A G L Q
F P V G R V H R L L R K G N Y A E R V G A G A P V
743.5750
b11 2+(Ac)
b5 (Ac)
b17 3+(Ac)
b17 3+
b5
b11 2+
b6
b3
343.1725
528.2898
563.3149
578.2064
588.6778
603.6859
608.3543
624.3791
654.4839
656.3473
680.4846
707.4964
731.2606
762.8232
771.1508
m/z

3.12.2. Isoelectric Focusing

1. Dilute 10 µL of the protein of interest (1 nmol or 31.5 µg for histones) in 90 µL of separation buffer 3 (see Note 9).
2. Load the sample into the separation chamber using a sample pump flow rate of 1 mL/h. Continue sample injection until the entire 100 µL volume has been loaded into the separation chamber. The medium introduction flow rate should be 180 mL/h.
3. Stop the sample pump after injection is complete.
4. Histone samples must be focused by cycling the flow rate forward for 3 min and then reverse for 3 min for a total of 40 min. The media pump flow rate during cycling should be 60 mL/h.
5. Begin collecting plate fractions into a microtest flat bottom 96-well plate, 17 min after sample injection is stopped. The medium flow rate during sample exit should be 180 mL/h. (see Note 10).
6. Collect two plates at 11 min per plate.
7. After collection, plates are frozen at –80°C or processed immediately using C 4 ZipTips™ (see Notes 10 and 11).

3.12.3. C4 Reversed-Phase Purification of IEF-FFE Fractions

1. Collect IEF-FFE fractions of interest into individual microfuge tubes.
2. Bind the sample to a C 4 ZipTip™ preequilibrated with 0.1% TFA.
3. Wash with 10 × 10 µL volumes of 0.1% TFA.
4. Elute with 5 µL 80% methanol, 1% acetic acid.
5. Freeze sample at –80°C or proceed to MS analysis.

3.13. FFE Combined with LC-MS

For increased resolution, FFE may also be combined with LC-FTICR-MS (Fig. 23). Not only does LC minimize ion suppression, but it also allows the full automation of the mass spectrometric process (as compared to direct-infusion FTICR-MS) (80). This makes LC-FTICR-MS ideal for detecting the components present in low abundance in a complex mixture. After performing FFE, histones are separated by type using RP separation. Each of these eluants is then individually examined to distinguish the different subtypes as well as the PTMs. PTMs are likely to influence the charge and, therefore, the way the protein migrates when subjected to electrophoretic separation methods. Although H1, H2A, H2B, H3, and H4 may have similar pIs and elute in the same FFE fraction, they each may have a distinct and very different hydrophobicity. H1 histones are the most difficult to ionize and therefore, the most difficult to detect by MS (80). Since FFE is the most laborious of the separation steps, it is recommended that it be performed prior to LC analysis so that FFE need to be conducted only once on the initial complex sample (rather than for multiple LC fractions).

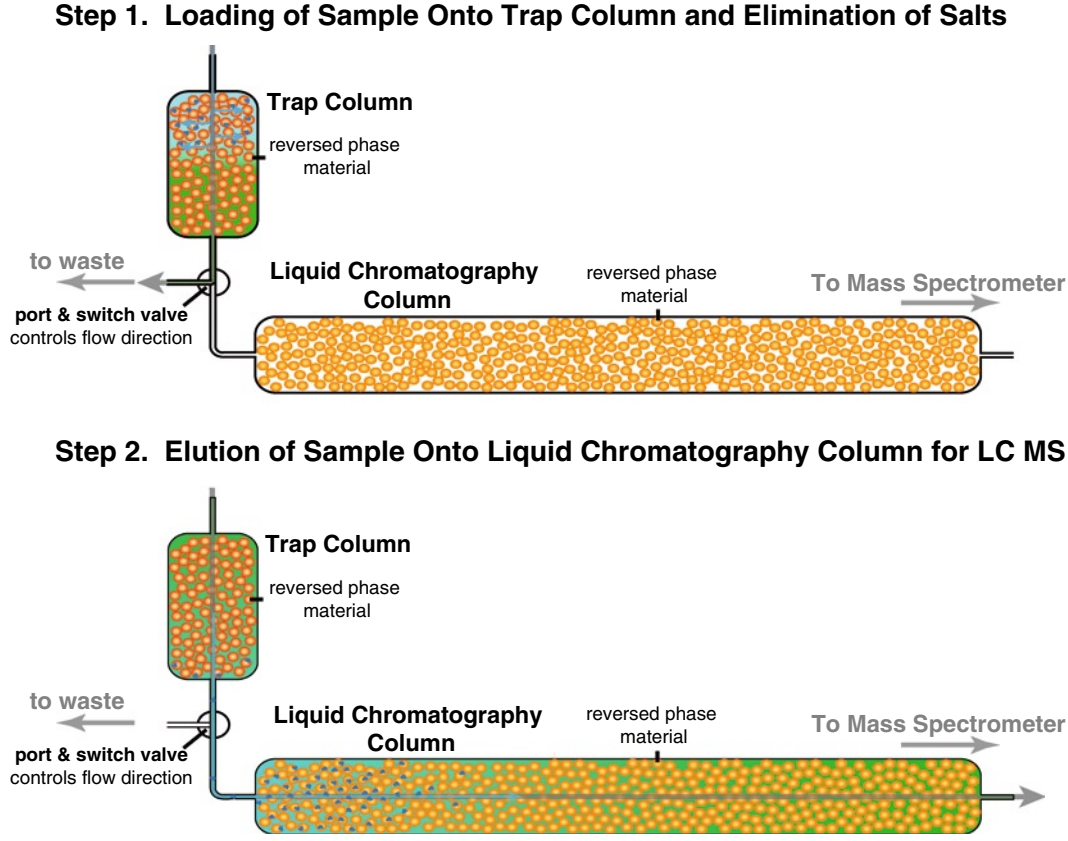

Step 1. Loading of Sample Onto Trap Column and Elimination of Salts
Trap Column
reversed phase material
to waste
port & switch valve controls flow direction
Liquid Chromatography Column
reversed phase material
To Mass Spectrometer
Step 2. Elution of Sample Onto Liquid Chromatography Column for LC MS
Trap Column
reversed phase material
to waste
port & switch valve controls flow direction
Liquid Chromatography Column
reversed phase material
To Mass Spectrometer

Fig. 23. Removal of salts from sample fractions and LC-MS. Some salts are not compatible for use with MS. Salts can contribute to ion suppression and reduced sensitivity. (1) The removal of salts may be achieved through the use of a reversed-phase trap column and washing with an appropriate solvent system prior to LC-MS analysis. The sample is retained on the column after washing while the salt is washed away. (2) The sample fraction can now be eluted off the trap column and onto the LC column to further separate the protein sample. The sample components travel through the reversed-phase column with the solvent and eventually elute off the LC column at different times according to their relative hydrophobicity. These eluted proteins then enter the mass spectrometer for analysis.

3.13.1. Post-FFE HPLC

1. Separate histone fractions (FFE fractions) on an UltiMate 3000 (Dionex, Sunnyvale, CA) HPLC system equipped with a 200 µm ID PS-DVB monolithic column (Dionex) (see Note 12).
2. Ensure that the preconcentration system (5-mm trap and 5-cm analytical column) is directly interfaced to the FTICR-MS with a custom 20 µm-ID interface, similar to the interface described previously by Toll et al. (81).

   This customization allows the application of the 2.5 µL/min flow rates with acceptable backpressure and no added extra-column volume.
3. Prior to injection, dilute samples (1:1) in the loading solvent (0.05% HFBA).

4. Load the sample onto the trapping column and wash for 3 min.
5. During loading, increase the ACN concentration to 25% in 2 min, followed by a 10 min gradient from 25 to 45% ACN. This gradient should be followed by a 4-min wash at 50% ACN and an 8-min equilibration at 0% ACN, resulting in a total analysis time of 25 min per sample.
6. Use a loading flow rate of 20 μL/min and an analytical flow rate of 2.5 μL/min.

3.13.2. FTICR-MS

1. For MS analysis of the intact histones, couple the LC system online to a 12-T Apex-Qe FT-ICR mass spectrometer.
2. Limit the scan range to m/z 650–1,200 (see Note 13).
3. Set the spectra accumulation to 1 to acquire a single scan without averaging (see Note 14).
4. Averaging can be performed post-acquisition using the data analysis software by averaging all spectra from scans across a chromatographic peak.

4. Notes

1. HPMC solutions should be discarded if not used within 2–3 weeks, as they are prone to bacterial growth.
2. Every instrument has default methods that may be modified by the user. A generic positive-mode MS method may be used as a starting point.
3. Laser intensity should be adjusted after acquisition of the first few spectra. These spectra should be used to gauge the sensitivity and mass accuracy of the instrument, which tend to fluctuate with environmental conditions. They also act as an indication of appropriate laser strength for the experiment.
4. Ammonium citrate can enhance the detection of modified peptides in MALDI-MS analysis (82). If enhanced detection is needed, add 0.5 μl of the resin, 0.5 μL of the matrix, and 0.25 μL of 25 mM ammonium citrate onto the MALDI spot and allow to dry at room temperature.
5. An approximately equimolar mixture of two peptides can be acetylated by addition of a 500-fold molar excess of acetic anhydride/amino group at a peptide concentration of 1 μg/μL.
6. If the absorbance reading (215 nm) is high enough (~1 AU or higher) for any fraction collected during the SCX, use a longer (~2.5 h) gradient (95–50% solvent A) to compensate for the higher peptide concentration in that fraction. This allows for sufficient peptide separation on the column.

7. When the sample is diluted in a high concentration of organic solvents (such as 80%), the mobile phase is kept at 50% organic, where the spray is more stable.
8. Coating with HPMC reduces electroendosmosis (83).
9. Before loading the protein, the system should be tested. This should be done by conducting a control experiment with a pI mix to verify proper isoelectric focusing.
10. Long-term exposure of protein to high urea concentrations can lead to carbamylation of lysine residues. This results in unfavorable heterogeneity in downstream top-down MS analyses. Since carbamylation is reduced in acidic buffers, pre-acidification is highly recommended for each well of the 96-well collection plates. To do this, use 20 μL of 10% TFA (final acid concentration after fraction collection is approximately 0.9%). Although this step is not necessary for samples that are further processed soon after plate collection, the acidification step does ensure that protein fractions are less susceptible to carbamylation over long periods of time and/or multiple freeze-thaw cycles.
11. For analysis of simple protein mixtures, it is useful to identify the protein-containing fractions by measuring the protein concentration of the collected plates using a Bradford (or similar) assay. Typically, 50 μL of each fraction is combined with 150 μL of 20% Bradford reagent in a 96-well format and the absorbance at 595 nm is measured using a microplate reader.
12. The use of monolithic stationary phases in HPLC columns for the removal of FFE matrix via RP has the added benefit of increased component separation and eliminates the problem of mass transfer in and out of the pores of conventional stationary phases.
13. A critical step in the analysis is the optimization of the scan parameters; the MS scan time must also be compatible with the average peak width of the compounds eluting from the monolithic column.
14. This allows a collection cycle of about 3 s. The spectral accumulation is set to a value of 1 so that we do not average/combine spectra that may belong to an adjacent chromatographic peak.

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Part VI

Spectroscopic and Whole Tissue Analyses

Chapter 17

In Vivo Nuclear Magnetic Resonance Metabolite Profiling in Plant Seeds

Victor Terskikh and Allison R. Kermode

Abstract

Nuclear magnetic resonance (NMR) spectroscopy has been successfully applied to profile a variety of primary and secondary metabolites in whole intact plant seeds in vivo. The nondestructive nature of NMR spectroscopy allows direct metabolic studies to be performed on the same seed throughout a given physiological process or key lifecycle transition, such as dormancy breakage, germination, and early postgerminative growth. Multinuclear NMR is capable of evaluating seed quality by assessing nondestructively nutrient reserves and seed protectants at seed maturity and to further monitor reserve mobilization following germination, which is critical for seedling emergence. In this chapter, we illustrate the use of several in vivo NMR techniques for metabolite profiling in seeds. Importantly, some of these methods have potential for the screening of single seeds or seed populations to identify seedlots with compromised viability either due to developmental problems or as a result of deterioration during prolonged storage.

Key words: Nuclear magnetic resonance, NMR, Magic angle spinning, MAS, Metabolite profiling, Germination, Imbibition, Seed deterioration

1. Introduction

Nuclear magnetic resonance (NMR) spectroscopy finds many applications in modern research environment, including plant biology and physiology (1–3). Of particular importance to this chapter, NMR has been successful in profiling noninvasively different classes of metabolites in vivo in living plants (4–6). In vivo NMR is a noninvasive and direct approach to tracing physiological processes in plant tissues and correlating them with metabolism. The main advantage of this technique is that it eliminates the need for metabolite extraction from plant tissues, a process that can result in alteration of metabolites.

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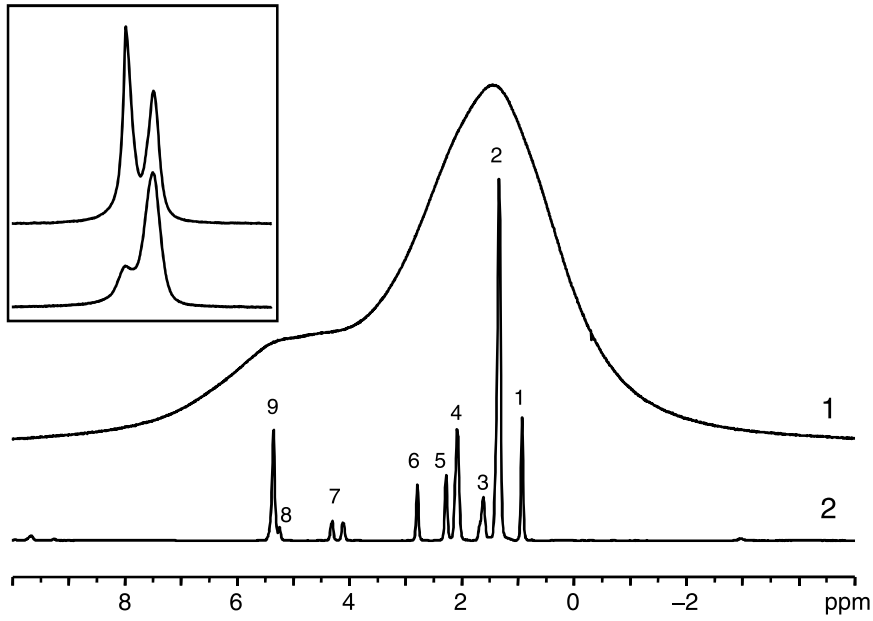

Fig. 1. In vivo ^{1}H NMR spectra of a single seed of western white pine (*Pinus monticola* Dougl. ex D. Don) showing static spectrum (**1**) and spectrum under moderate magic angle spinning (MAS, 3 kHz) (**2**). Individual resonances in the MAS spectrum shown in (**2**) are assigned as follows (1) $-CH_3$; (2) $-(CH_2)_n-$; (3) $-COC\mathbf{H}_2CH_2-$; (4) $-HC=CHC\mathbf{H}_2-$; (5) $-COCH_2-$; (6) $-HC=CH-C\mathbf{H}_2-HC=CH-$; (7) $-CH_2O-$; (8) $-CHO-$; (9) $-HC=CH-$. *Inset* shows static ^{1}H NMR spectra of a dry seed (*lower*) and a seed fully imbibed (soaked in water for 12 days at 23°C) (*upper*). Chemical shift scale is in parts per million (ppm) referenced to external tetramethylsilane (TMS). Adapted from (14) with permission from Oxford University Press.

In plant seeds, multinuclear in vivo NMR is capable of detecting and identifying several primary and secondary metabolites. For example, ^{1}H and ^{13}C NMR has been successfully used for the qualitative and quantitative nondestructive analysis of storage oils [triacylglycerols (TAGs)] in a variety of angiosperm and gymnosperm seeds, including those of canola (7, 8), soybean (9), lettuce, Arabidopsis, garden pea (10, 11), sunflower (7, 12), silver fir (*Abies alba* Mill.) (13), and other coniferous seeds (14, 15).

Examples of ^{1}H NMR spectra recorded in vivo in single seeds of western white pine (*Pinus monticola*) are shown in Fig. 1. A static spectrum of a single dry mature seed consists of two broad overlapping peaks: one at 1–2 ppm from methyl/methylene protons in hydrocarbon chains of fatty acids, and the other at 4–5 ppm from olefin protons and a glycerol moiety (Fig. 1, 1). These two broad peaks are resolved into many individual resonances under moderate magic angle spinning (MAS) (Fig. 1, 2). Although static ^{1}H NMR spectra lack sufficient resolution for detailed analysis, such spectra could be useful to study seed imbibition via monitoring the water resonance at 4.5 ppm (Fig. 1, inset) (16). Spatial distribution of water and oil in seeds can be visualized using in vivo ^{1}H NMR microimaging (Chapter 18).

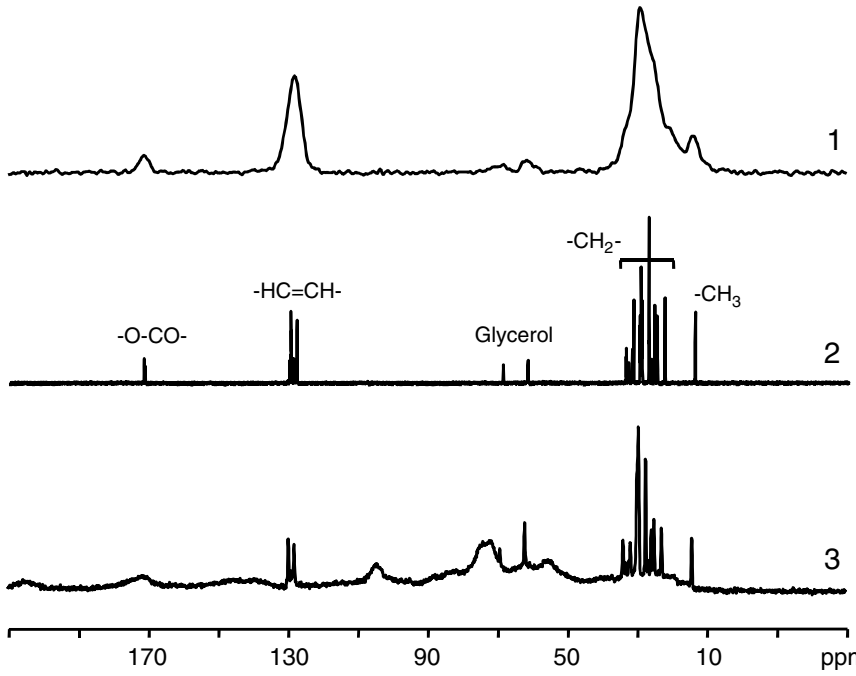

Fig. 2. Three different ^{13}C NMR techniques which can be employed to study plant seeds in vivo in relation to oils. (**1**) Acquisition under stationary conditions with low-power broadband proton decoupling. The spectrum shown was recorded for 0.1 g (about 80 seeds) of dry mature western redcedar (*Thuja plicata* Donn ex D. Don) seeds. (**2**) Acquisition under moderate magic angle spinning (MAS, 3 kHz) with low-power broadband proton decoupling. The spectrum shown was recorded for a single-dry seed of western white pine (*Pinus monticola* Dougl. ex D. Don). (**3**) The same seed as in (2), the spectrum acquired under ^{13}C cross-polarization conditions (CP/MAS) and high-power proton decoupling. Chemical shift scale is in parts per million (ppm) referenced to external tetramethylsilane (TMS). Adapted from (14) with permission from Oxford University Press.

Seeds of many species contain storage oils (TAGs) as a major nutrient reserve to support early seedling growth following germination. In the storage tissues of seeds, the TAGs are stored in oil bodies and remain liquid even at the low water content of mature dry seeds. This unique feature of storage oils has proven useful in studying them with ^{13}C NMR spectroscopy. The static ^{13}C NMR spectra consist of several broad resonances originating from different types of carbon atoms in the seed oils (Fig. 2, 1). A possible correlation between the intensity of the static ^{13}C NMR line from unsaturated carbon atoms of storage oils and the germination capacity of western redcedar seeds was reported (15). Such spectra still lack high resolution; the broadening of peaks is caused by magnetic susceptibility effects and residual dipolar interactions (9, 11–13). Considerably improved spectral resolution of oilseed lipids can be achieved under MAS conditions (7, 8, 17). Even moderate MAS eliminates undesirable line broadening and results in excellent spectral resolution (Fig. 2, 2), comparable with high-resolution NMR spectra of true liquids. ^{13}C MAS NMR spectra of dry

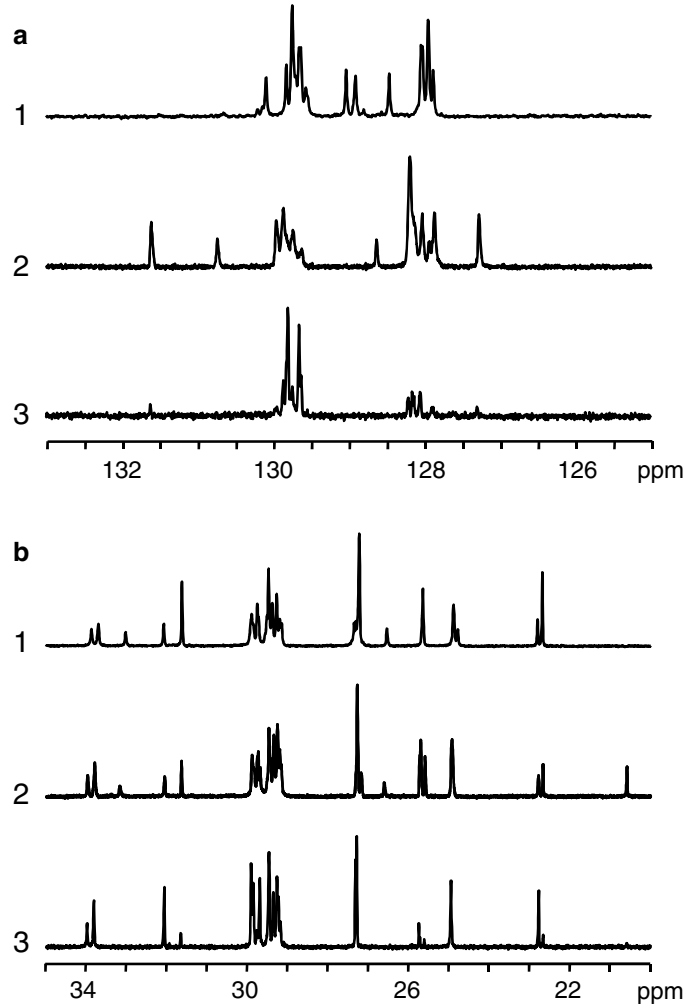

Fig. 3. In vivo ^{13}C MAS NMR spectra of dry quiescent seeds of three plant species illustrating variability in the fatty acid constituents of the storage oil reserves. (**a**) The olefin carbon region of the spectra. (**b**) The methylene carbon region of the spectra. (1) Western white pine (*Pinus monticola* Dougl. ex D. Don). The major fatty acids are 9–18:1, 9,12–18:2, and 5,9,12–18:3. (2) Western redcedar (*Thuja plicata* Donn ex D. Don). The major fatty acids are 9–18:1, 9,12–18:2, 9,12,15–18:3, and 5,11,14,17–18:4. (3) Rapeseed (*Brassica napus*). The major fatty acids are 9–18:1, 9,12–18:2, and 9,12,15–18:3. Chemical shift scale is in parts per million (ppm) referenced to external tetramethylsilane (TMS).

oil seeds consist primarily of two groups of lines from hydrocarbon chains of fatty acids: one group derives from olefin carbon atoms at 125–132 ppm (Fig. 3a), and the other group derives from aliphatic carbon atoms at 21–35 ppm (Fig. 3b). Solitary lines are from the fatty acid terminal methyl groups, triglyceride backbone carbons, and carboxyl carbons. Such highly resolved spectra allow in vivo identification and quantification of major fatty acids (Table 1).

Table 1
Assignment of ^{13}C NMR MAS resonances to oleic (9–18:1), linoleic (9,12–18:2) and pinolenic (5,9,12–18:3) fatty acids in seed storage oils of Pinaceae family

$\delta^{13}C$ (ppm)	9–18:1	9,12–18:2	5,9,12–18:3
C-18	14.04	14.00	14.00
C-17	22.78	22.66	22.66
C-16	32.06	31.60	31.60
C-15	29.1–29.9	29.1–29.9	29.1–29.9
C-14	29.1–29.9	27.2–27.4	27.2–27.4
C-13	29.1–29.9	129.73	130.08
C-12	29.1–29.9	127.93	127.87
C-11	27.2–27.4	25.60	25.60
C-10	129.68	128.00	128.44
C-9	129.53	129.62	129.02
C-8	27.2–27.4	27.2–27.4	27.2–27.4
C-7	29.1–29.9	29.1–29.9	27.2–27.4
C-6	29.1–29.9	29.1–29.9	129.81
C-5	29.1–29.9	29.1–29.9	128.90
C-4	29.1–29.9	29.1–29.9	26.51
C-3	24.86	24.86	24.76
C-2 (*sn*-1,3)	33.67	33.67	33.00
C-2 (*sn*-2)	33.85	33.85	33.85
C-1 (*sn*-1,3)	171.72	171.72	171.68
C-1 (*sn*-2)	171.46	171.46	171.46

^{13}C NMR has been used as a means of monitoring the mobilization of oil reserves following seed germination and during early seedling growth in vivo, for example, in seeds of soybean (18, 19), lodgepole pine, Sitka spruce (17), and western white pine (14). Protein and carbohydrate reserves can also be monitored in seeds in vivo using ^{13}C MAS (9, 20–22). In addition, a number of monoterpenes are identified in the ^{13}C NMR spectra of conifer seeds containing oleoresin (14). In imbibed conifer seeds, the ^{13}C MAS NMR spectra show the presence of considerable amounts of dissolved sucrose presumed to play a protective role in the desiccation tolerance of seeds (14, 17). The free amino acids, arginine and

asparagine, generated as a result of storage protein mobilization, are detected in vivo during conifer seed germination and early seedling growth (14).

^{31}P MAS NMR spectroscopy is less common in metabolic studies in plant seeds; however, it can also be utilized to profile nondestructively a variety of phosphorus-containing compounds in mature and germinating seeds, including phytates, phospholipids, nucleic acids, and inorganic P compounds (19, 21, 23–25). For example, in the mature dry western white pine seed the ^{31}P MAS NMR spectrum represents a set of broadened lines separated by 3 kHz (MAS spinning speed) (Fig. 4, 1). Of these lines only one does not change its position when different spinning speeds are used – the central line. The appearance of this spectrum resembles that of phytates, major phosphorus storage constituents of most cereal, legume, and oil seeds. When dormant western white pine seeds are imbibed, some phytate dissolves giving rise to a narrow isotropic line at about 0 ppm (Fig. 4, 2). Other phosphorus-containing

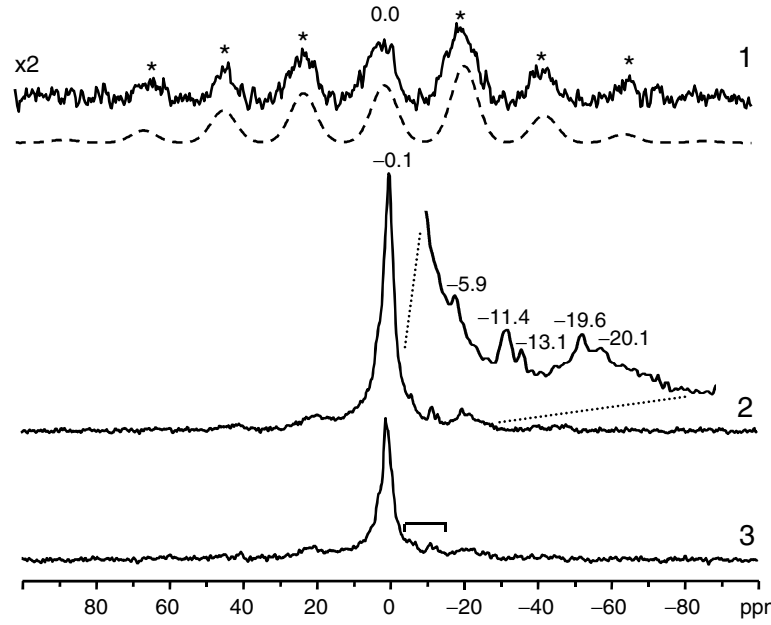

Fig. 4. In vivo ^{31}P MAS NMR spectra of western white pine (*Pinus monticola* Dougl. ex D. Don) seeds. (**1**) A single-mature dry seed. The spinning side-bands that are due to 3 kHz MAS are marked with the asterisks. The isotropic resonance is at 0.0 ppm. The lower trace is a calculated spectrum (see text). (**2**) A single fully imbibed seed (i.e., a seed soaked in water for 12 days at 23°C). Individual resonances are assigned as follows: (–0.1 ppm) phytate, sugar mono and diphosphate, vacuolar and cytoplasmic P_i (inorganic phosphate), (–5.9 ppm) γ-NTP, (–11.4 ppm) α-NTP, UDPG and NAD(P)H, (–13.1 ppm) UDPG, (–19.6 ppm) β-NTP, (–20.1 ppm) polyphosphate. (**3**) A single germinated seed. The seed was subjected to a 12-day running water soak, moist-chilled for 3 months and then germinated at daylight regime at 23°C for 12 days. Relative intensities of three spectra are preserved. Chemical shift scale is in parts per million (ppm) referenced to external 85% H_3PO_4.

species contributing to the line at 0 ppm are likely sugar phosphates (such as glucose-6-monophosphate and fructose-1,6-diphosphate) and vacuolar and cytoplasmic P_i (inorganic phosphate) of the liquid phase. The resonances from trinucleotides can be seen to the right of the main line overlapping with uridine diphosphoglucose (UDPG) and NADPH signals. Following dormancy breakage and germination of the seeds, NTP signals can also be seen in the spectrum (Fig. 4, 3). The overall intensity of the latter spectrum is diminished compared with the dormant-imbibed seed, since P-containing species (mostly phytate) are being mobilized and utilized by the growing seedling for phospholipid membrane synthesis and other metabolic processes.

We present herein the most typical hardware setup and experimental requirements for the straightforward acquisition of multinuclear NMR spectra in plant seeds in vivo. These requirements may vary depending on the particular research goal and the availability of equipment. In addition, we present in Table 2, a

Table 2
In vivo NMR-based metabolite profiling in plant seeds

nucleus	NMR method	Metabolites detected	Area of research
^{1}H	Static	Water Total lipids (TAGs)	Seed development; lipid accumulation; seed moisture content; seed deterioration during storage; seed imbibition and early germination
	MAS	Water Fatty acids Dissolved sugars	TAG composition; iodine value; markers for seed deterioration during storage; lipid reserve mobilization during postgerminative growth; and water status and mobility
^{13}C	Static	Total lipids (TAGs)	Storage oil content and composition; iodine value; markers for seed deterioration during storage; lipid oxidation; and lipid reserve mobilization during postgerminative growth
	MAS	Triglycerides Soluble sugars Amino acids Monoterpenes	Detailed composition of fatty acids; *sn*-positional isomers; seed development; seed deterioration during storage; lipid, protein, and carbohydrate reserve mobilization during postgerminative growth; primary metabolites; and secondary metabolites related to defense or another function
^{31}P	MAS	NTPs/NDPs Phytate Sugar mono- and diphosphate Vacuolar and cytoplasmic inorganic phosphate Polyphosphate	Seed development; phytate accumulation as a primary phosphorus/mineral storage reserve; phytate mobilization during postgerminative growth; and NTP/NDP turnover during and following germination as a measure of seedling vigor

summary of NMR-based methods, the molecules they detect, and the importance of the NMR tool to the study of a specific aspect of seed quality.

2. Materials

1. Mature seeds of western white pine (*Pinus monticola* Dougl. ex D. Don), western redcedar (*Thuja plicata* Donn ex D. Don), and other coniferous species. In our case, these were supplied by the Tree Seed Centre, British Columbia Ministry of Forests, Surrey, BC, Canada. The dry seeds (moisture content 5–7% on fresh weight basis) must be stored at –20°C.
2. To obtain fully imbibed seeds of western white pine, dry seeds are soaked in running water at 23°C for 12 days. To break dormancy, imbibed pine seeds are subjected to 3 months of moist chilling, and germinated at daylight regime at 23°C for 12 days as described elsewhere (26) and in this book (Chapter 4).

3. Methods

3.1. NMR Spectroscopy

NMR spectra of seeds in vivo can be recorded using any commercial NMR spectrometer (see Note 1). In our case, we utilized an Avance NMR spectrometer manufactured by Bruker BioSpin, which is located at the NMR Facility of the National Research Council Canada – Plant Biotechnology Institute, NRC–PBI (Saskatoon, Canada). This spectrometer is equipped with a wide-bore 8.5 Tesla Oxford superconducting magnet operating at the proton resonance frequency of 360.13 MHz. We describe below two different types of NMR experiments that are employed to obtain low-resolution NMR spectra in stationary seed samples and high-resolution NMR spectra with samples under MAS (see Note 2).

3.2. NMR Experiments with Stationary Seed Samples

1. Low-resolution static NMR spectra of seeds are acquired using a liquid-state 10 mm double-tuned $^{1}H/^{13}C$ NMR probe (see Note 2). A known amount of seeds without any additional treatment, typically 0.1 g, are placed in a 10-mm NMR glass tube and ^{1}H and ^{13}C NMR spectra are recorded. For quantitative NMR measurements, three or more replicates of 0.1 g seeds can be used. These experiments can also be performed with a single seed.
2. ^{1}H NMR spectra are recorded at a resonance frequency of 360.13 MHz with a spectral width of 50–70 kHz applying a

single-pulse sequence with a 5 μs radio frequency (RF) pulse and a 5 s relaxation delay between scans. Usually, from eight to thirty-two transients are accumulated for each ^{1}H NMR spectrum. ^{1}H NMR chemical shifts are referenced to external tetramethylsilane (TMS).

3. ^{13}C NMR spectra are obtained at a resonance frequency of 90.56 MHz with a spectral width of 20–25 kHz under low-power broadband proton decoupling and ^{13}C pulse width of 10 μs with a 5 s relaxation delay between scans. The typical number of accumulations is 1024. ^{13}C NMR chemical shifts are referenced to external TMS.

3.3. NMR Experiments with Samples Under MAS

1. High-resolution NMR spectra of plant seeds are recorded with solid-state double-tuned $^{1}H/X$ or $^{1}H/^{13}C$ MAS probes (see Note 3). Smaller seeds are placed in a 4 mm o.d. ZrO_2 rotor and spun under the magic angle at a spinning speed of 3 kHz. Larger seeds are placed in a 7 mm o.d. ZrO_2 rotor, additionally packed with glass beads to achieve better balance and spun under magic angle at 3 kHz (see Note 4).
2. ^{1}H MAS NMR spectra are recorded with a spectral width of 50–70 kHz applying a single-pulse sequence with a 5 μs RF pulse and a 5 s relaxation delay between scans. Time domain size of the spectra is 4 K with eight accumulations per spectrum. ^{1}H NMR chemical shifts are referenced to external TMS.
3. ^{13}C MAS NMR spectra are obtained with a spectral width of 20–25 kHz under low-power proton decoupling and ^{13}C pulse width of 3 μs and 1 s relaxation delay between scans. Time domain size of the spectra is 32 K. The number of accumulations is 4096. ^{13}C NMR chemical shifts are referenced to external TMS.
4. ^{31}P MAS NMR spectra are obtained at a resonance frequency of 145.78 MHz with a spectral width of 50 kHz under a broadband high-power proton decoupling. Dry, imbibed or germinated seeds are placed in a 7 mm o.d. ZrO_2 rotor, additionally packed with glass beads to achieve better balance and spun under magic angle at 3 kHz. Spinning side-bands are identified in the spectra of the sample spinning at 2.5, 3.5, and 4 kHz. Time domain size of the spectra is 1 K with no zero-filling and 50 Hz exponential multiplication before Fourier transformation. Number of accumulations are 32,768 (32 K) with a relaxation delay of 1 s and ^{31}P $\pi/4$ pulse width of 3 μs. Phosphorus chemical shifts are referenced to external 85% H_3PO_4, with a polycrystalline $NH_4H_2PO_4$ sample used as a secondary chemical shift reference (isotropic ^{31}P chemical shift at 0.8 ppm).

4. Notes

1. For the purposes of in vivo NMR metabolite profiling in plant seeds, NMR spectrometers in a variety of configurations can be used, with the final choice depending mostly on the immediate availability. It is recommended, however, to use NMR instruments with a moderate magnetic field strength, i.e., with the proton resonance frequency ranging from 200 MHz (4.7 T) to 400 MHz (9.4 T), although both the lower and the higher magnetic fields may also be successfully employed. Regardless of the magnetic field strength, an NMR instrument itself should be equipped according to the type of experiments to be performed. Most of the experiments outlined above can be executed on a spectrometer configured for liquid-state NMR research, i.e., outfitted only with low-power (100 W) radio frequency amplifiers. Such instruments can be found in practically any modern analytical facility performing routine NMR experiments.
2. An NMR probe is another important hardware item to keep in mind while planning NMR experiments. Generally, two types of probes should be considered. It is the most straightforward for low-resolution NMR experiments with stationary samples to use widely available standard liquid-state NMR probes equipped with two separate channels tuned to proton (^{1}H) and carbon (^{13}C) resonance frequencies. Typically, no deuterium lock channel is required or any other means of magnetic field stabilization.
3. For high-resolution NMR experiments with samples spinning under the MAS, it is necessary to use more specialized MAS NMR probes and pneumatic equipment normally required for solid-state NMR research. The MAS rotor size is not critical from the hardware side as only very moderate MAS speeds are employed, generally no faster than 2–3 kHz. However, the rotor size becomes important when considering the seed size (see below). Although MAS NMR probes are normally used with the high-power radio frequency amplifiers, for most in vivo MAS NMR experiments in seeds an NMR instrument should be still configured for acquisition of the spectra in the low-power mode, as it is done in liquid-state NMR experiments. One should consult with an NMR Facility specialist before performing any experiments. Improper instrument setup could lead to instrument malfunction or equipment damage.
4. Plant seeds vary greatly in size among species, and even within the same species or within the same seedlot ranging from millimeters to centimeters. While of little importance for ex vivo experiments with seed extracts, this variability in seed size may

potentially limit the range of available NMR experiments to be performed in vivo. The size limitation is less important when NMR spectra are recorded for stationary seed samples using NMR probes designed for liquid-state NMR spectroscopy. A typical sample container used with these probes is a traditional glass NMR tube with an external diameter of 5 or 10 mm. This size is sufficient to accommodate seeds of many plant species. To accommodate larger seeds, NMR probes with glass tubes up to 20 mm in diameter may be available at some NMR Facilities. The seed size consideration becomes more important when recording high-resolution NMR spectra with samples spinning under the MAS. In this case, seeds should fit inside an MAS rotor, and would rarely exceed about 5 mm in size.

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Chapter 18

In Vivo ^{1}H-NMR Microimaging During Seed Imbibition, Germination, and Early Growth

Victor Terskikh, Kerstin Müller, Allison R. Kermode, and Gerhard Leubner-Metzger

Abstract

Magnetic resonance imaging (MRI) is a superior noninvasive diagnostic tool widely used in clinical medicine, with more than 60 million MRI tests performed each year worldwide. More specialized high-resolution MRI systems capable of a resolution that is 100–1,000 times higher than standard MRI instruments are used primarily in materials science, but are used with increasing frequency in plant physiology. We have shown that high-resolution ^{1}H-nuclear magnetic resonance (NMR) microimaging can provide a wealth of information about the internal anatomy of plant seeds as small as 1 mm or even smaller. This chapter covers the methods associated with these imaging techniques in detail. We also discuss the application of ^{1}H-NMR microimaging to study in vivo seed imbibition, germination, and early seedling growth.

Key words: Nuclear magnetic resonance, Magnetic resonance imaging, Microimaging, Chemical shift selective imaging, Seed imbibition, Germination

1. Introduction

Water uptake is a fundamental requirement for the initiation and completion of seed germination (1, 2). "Dry" orthodox seeds usually have water potentials between –350 and –50 MPa corresponding to a moisture content of only 5–10%. Water has by definition a water potential of 0 MPa, and leaf tissue has a water potential around –1 MPa. The water potential of plant tissues or of soil is determined by the sum of their pressure potential (a positive hydrostatic or turgor pressure), osmotic potential (a negative osmotic pressure), and matric potential (a negative value, important for "dry" states). Uptake of water by a "dry" seed is triphasic with a rapid initial uptake (phase I, i.e., imbibition) followed by a plateau

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phase (phase II). A further increase in water uptake occurs only after germination is completed, as the embryo axis elongates after having emerged from all the seed-covering layers. Because dormant seeds do not complete germination, they do not enter this postgermination phase of water uptake (phase III). Abscisic acid (ABA) inhibits phase III water uptake and the transition from germination to postgerminative growth (3–5). While the temporal pattern of water uptake by seeds has been well-studied in different species, far less is known about its spatial distribution within seed tissues and how it is affected by plant hormones, environmental cues, and seed-covering layers/coats.

^{1}H-nuclear magnetic resonance (NMR) imaging (MRI) is a noninvasive in vivo technique that allows the acquisition of sequential cross-sectional two-dimensional (2D) and three-dimensional (3D) images of the spatial distribution of ^{1}H nuclei (mobile protons) and as such has been applied to plant biology (e.g., (3, 6–10)). The underlying principle of ^{1}H-NMR is that the ^{1}H nuclei carry a nonzero spin and, therefore, a magnetic moment. NMR signals occur if a sample with ^{1}H nuclei is immersed in a strong, external, static magnetic field (B_0) and is exposed to energy delivered by radio frequency pulses (11). The interaction of the magnetic moment with the field B_0 causes the nuclear spins to align and thus creates a small magnetization vector within the sample. The application of the radio frequency pulses of a given power and duration manipulates this magnetization. After the application of the radio frequency pulse, the magnetization vector spins around B_0 with its specific Larmor frequency, which is proportional to the field strength. For ^{1}H nuclei at a magnetic field strength B_0 of 9.4 T, the Larmor frequency is 400 MHz. NMR imaging is based on the fact that the Larmor frequency is proportional to the polarizing magnetic field. If in addition to B_0 a uniform magnetic field gradient G is applied, this frequency experiences a spatial signature and its position can be calculated. The interaction of the magnetic moment with externally applied magnetic field gradients and radio frequencies is used to obtain two-dimensional or three-dimensional images. In cases where the resolved volume elements are finer than the resolution of the unaided human eye (approx. 0.1 mm), this method of imaging may be termed microscopic or microimaging (12). A recent review by Stark et al. (11) further describes these techniques.

^{1}H-NMR imaging and microimaging have been used to study water uptake and flow during germination in relatively large seeds/fruits of angiosperms and gymnosperms, including those of legumes (13–16), conifers (17–19), cereal grains (11, 20–26), and others (27). Redistribution of water and tissue-specific differences in moisture content and water entry points were detected in these seeds. For example, in developing spruce (*Picea glauca*) seeds, water was concentrated at the radicle pole (17). In western white

pine (*Pinus monticola*) seeds, imbibition is characterized by water penetration through the seed coat and megagametophyte (18). The cotyledons of the embryo (located in the chalazal end of the seed) are the first to show hydration followed by the hypocotyl and later the radicle. After penetrating the seed coat, water in the micropylar end of the seed likely also contributes to further hydration of the embryo; however, the micropyle itself does not appear to be a site for water entry into the seed (18). The endosperm is a prominent structure in cereal caryopses, and it shows certain specific characteristics in the spatial pattern of water uptake. In cereal grains, the seed coat and the embryo–scutellum region appear to be barriers to water uptake by the embryo (21–24). Prior to the completion of germination, water must accumulate in the embryo–scutellum tissues for redistribution to the endosperm to take place. Delayed or protracted hydration severely limits germination. Dissection studies of cereal seeds show that the hydration properties of seed tissues differ and that the endosperm can act as a water reservoir under water-limiting conditions (22, 24).

MRI experiments with tobacco seeds illustrate how microimaging can be successfully applied to seeds smaller than 1 mm in size (3, 28). A time course analysis of tobacco seed germination exhibits a "two-step" pattern: testa rupture (Fig. 1, top) precedes endosperm rupture (Fig. 1, bottom), which is the visible completion of germination (radicle protrusion). NMR microimaging confirms that water distribution in phase II and phase III tobacco seeds is not homogeneous. The micropylar seed end, where endosperm rupture occurs, appears to be the major entry point of water. The micropylar endosperm and the radicle show the highest hydration.

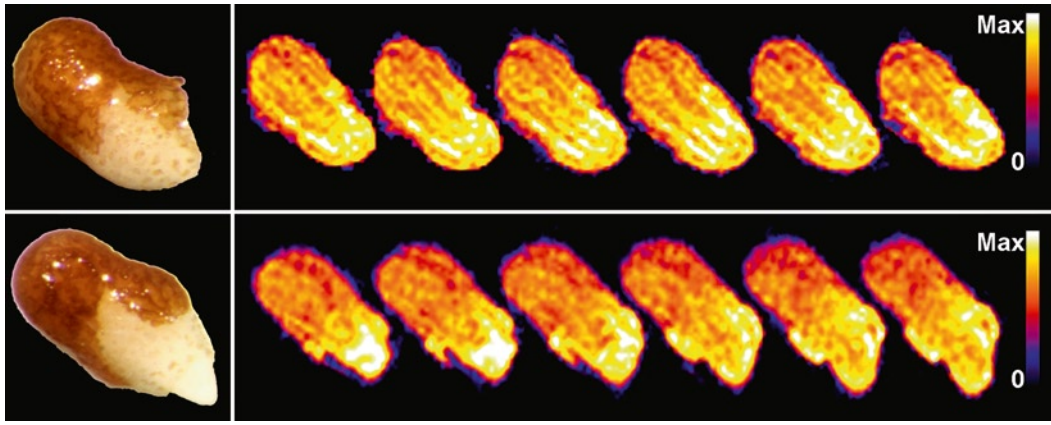

Fig. 1. Noninvasive in vivo ^{1}H-NMR microimaging of water uptake and distribution during tobacco seed germination. The spatial distribution of protons within the seed tissues is visualized with false colors as shown. The NMR microimages were obtained with 30 μm spatial resolution. Also shown are corresponding microphotographs of seeds in the testa rupture (*top left*) and endosperm rupture (*bottom left*) stages. Modified from Manz et al. (3), http://www.plantphysiol.org, Copyright American Society of Plant Biologists.

This spatial pattern of water uptake is already evident during the early phase of imbibition before visible testa rupture. It becomes even more pronounced during testa rupture and remains pronounced after endosperm rupture. These results intimate that the radicle and the micropylar endosperm of the tobacco seed have enhanced water-holding capacities and can serve as a water reservoir for the embryo during germination. The inhibition by ABA of tobacco endosperm rupture and phase III water uptake does not appear to involve an effect of ABA on the spatial distribution of water in the seed. Testa rupture and initial embryo elongation cause an additional increase in water uptake in the second half of phase II. The additional increase in water content in the late part of phase II of water uptake is also evident in ABA-treated seeds, which supports the contention that initial embryo elongation and water uptake by the micropylar endosperm are not inhibited by ABA. Water uptake by dormant tobacco seeds (29) is likewise congruent. Germination of these seeds is blocked before testa rupture and there is no additional increase in water content in late phase II of water uptake. ABA, therefore, inhibits germination and phase III of water uptake by the emerging embryo, but it does not inhibit phase II water uptake needed for initial embryo elongation. Further, its inhibitory action is not mediated by reducing the water-holding capacity of the micropylar endosperm (3).

Overall, these studies are important for understanding the germination characteristics of seeds; different seed tissues and organs hydrate at different extents and show different spatial characteristics of water distribution, and the micropylar endosperm of some seeds, such as tobacco, acts as a water reservoir for the embryo.

Dry tobacco seeds contain approximately 43% of triacylglycerols (TAGs) per fresh weight as the most abundant nutrient storage reserve (3, 28). In oilseeds, like tobacco, the TAGs are stored in oil bodies and are genuinely liquid at room temperature, thus contributing to ^{1}H-NMR microimages due to high proton mobility. The MRI images of phase I tobacco seeds represent a combination of the spatial contents of TAGs and water. The proton mobility becomes essentially specific for water during phase II as in this phase the TAG peaks are very small compared to the water peak (see Note 1).

Since water and oil NMR signals are well-separated by their chemical shifts, it is possible to obtain separate water- or oil-specific MRI images using a chemical shift selective imaging (CSSI) technique as illustrated in Fig. 2 for western white pine seeds (also see ref. 18). In imbibed pine seeds, mobile water is mainly concentrated in the embryo and the seed coats while both the megagametophyte and the embryo show high concentrations of storage oils. ^{1}H-NMR CSSI microimages of a germinating seed clearly show that the initiation of postgerminative reserve mobilization across the megagametophyte storage tissue and the embryo exhibits

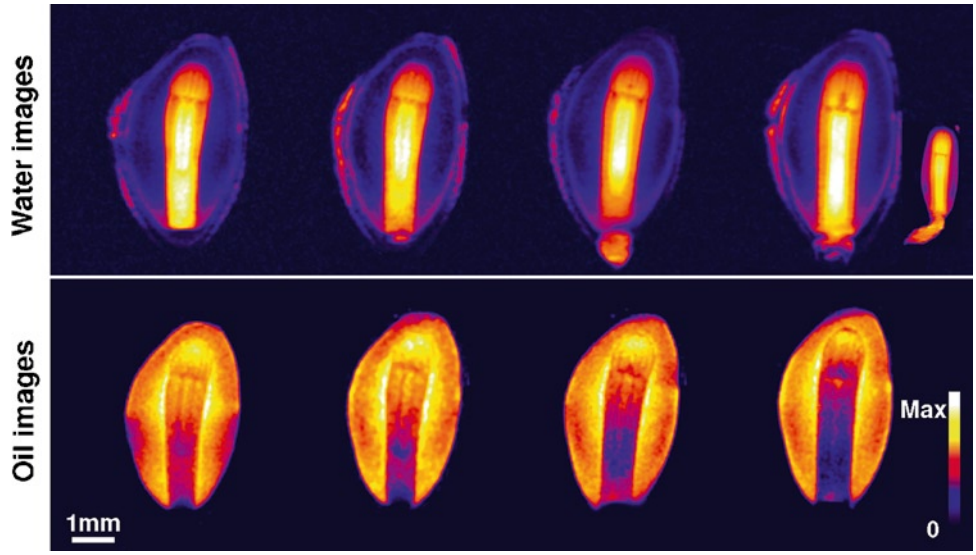

Fig. 2. In vivo ^{1}H-NMR chemical shift selective imaging (CSSI) of western white pine seed during germination. The spatial distribution of water (*upper row*) and oil (*lower row*) simultaneously mapped in the same seed over a course of early germination (*left* to *right*) is visualized with false colors as shown. Coronal plane 2D MRI images were obtained with a 78 μm in-plane resolution and a 500-μm slice thickness. For more information on experimental details, see ref. 18.

distinct regional (i.e., spatial) differences. Mobilization of the major lipid storage reserves commences first within the meristematic region of the embryo and is likely important for early seedling establishment (Fig. 2). In the megagametophyte (which generally comprises the bulk of the seed), the oil mobilization commences only after radicle and cotyledon elongation/expansion, i.e., it is a postgerminative event. Thus, the chemical shift selective ^{1}H-NMR microimaging allows direct in vivo monitoring of oil reserve mobilization during early postgerminative growth, a process upon which seedling emergence depends.

2. Materials

1. Seeds of *Nicotiana tabacum* (tobacco), *Pinus monticola* (western white pine), or other species.
2. Autoclaved distilled water or a weak salt medium, e.g., 1/10 Murashige and Skoog (MS) salts without organic additions (4).
3. Plastic Petri dishes, filter paper, and a growth chamber or incubator that can be maintained at a constant temperature and light intensity.
4. Bruker Avance 400 NMR spectrometer (Bruker, Rheinstetten, Germany) with a vertical 9.4 T magnet and a proton resonance

frequency of 400 MHz (8, 30) as per the NMR experiments conducted on tobacco seeds (3).

5. Bruker Avance DRX 360 spectrometer equipped with a Bruker 2.5 microimaging system as per the NMR experiments conducted on pine seeds (18).

3. Methods

3.1. Seed Germination

1. Tobacco seeds are sown in plastic Petri dishes containing filter paper wetted with water and incubated at a constant temperature around 20°C in continuous white light. Other species may require different germination temperatures and a previous dormancy-breaking treatment.
2. Dry seeds of western white pine (*Pinus monticola* Dougl. ex D. Don) and dry seeds are first soaked in running water at 23°C for 12 days prior to subjecting them to a subsequent prolonged moist-chilling treatment as described in Chapter 4 and in (31). Germination conditions are also described in (31).
3. The seeds are sampled after different time periods after imbibition of dry seeds (tobacco) or after seeds have been transferred into germination conditions after dormancy breakage (western white pine). The time course of germination must be monitored. For example, for tobacco seeds, one scores the percentage of testa and endosperm rupture over time in order to correlate the ^{1}H-NMR microimaging results with defined seed stages during germination (Fig. 1) (see Note 2).

3.2. In Vivo ^{1}H-MAS NMR Spectroscopic Analyses of Seed Moisture

1. For magnetic angle spinning (MAS) NMR spectroscopy, the intact seeds (dry seeds or seeds sampled after imbibition) are placed inside a standard 4-mm MAS o.d. rotor (3). The rotation rate is set to 10 kHz, which has no influence on the seed morphology; due to the small rotor diameter, the centrifugal forces are low. Also see Chapter 17.
2. The peak areas below the water signals at 4.8 ppm are quantified, and the relative moisture contents per seed are calculated as described in (3).
3. For comparison, gravimetric determination of seed moisture contents in mg of water per seed can be obtained. For this, the samples are weighed before and after heating for 3 h at 100°C as described in (32).

3.3. In Vivo ^{1}H-NMR Microimaging

1. Intact seeds are placed inside a fitting glass capillary, e.g., 1.5 mm in diameter for tobacco (3), and, in order to avoid movement during the experiment, are pressed slightly against

each other with a piece of matching Teflon tube at each end of the glass tube (see Note 3).

2. The capillary secured in an MRI probe, which is then positioned inside the superconducting magnet. There is no external movement of the sample tube similar to the slow spinning used in high-resolution NMR spectroscopy.
3. MRI images of the proton density are acquired with a Bruker Micro2.5 microimaging hardware setup using a standard three-dimensional spin-echo pulse sequence employing echo times (TE) of 1 ms, recycle delays (TR) of 0.8 s, spectral width of 100 kHz, and an isotropic spatial resolution of $30 \times 30 \times 30$ μm^3.
4. Each image consists of $128 \times 64 \times 64$ data points, and at least two signal averages should be taken. The total acquisition time is 110 min per image for tobacco seeds (3) (see Note 4).
5. The experimental MRI conditions may vary depending on the seed size and the spatial resolution required. For example, for larger seeds, two-dimensional MRI images can be acquired with a slice thickness of 0.2–0.5 mm.
6. CSSI can be conducted with a broadline imaging package (BLIP) developed by Bruker which allows simultaneous acquisition of both water and oil images. In CSSI experiments, refocusing and selection pulses are 1.2 and 0.3 μs, respectively, with an echo time (TE) of 4-8 μs and a recycle delay (TR) of 1 s.
7. False-color images of slices can be obtained from raw data with image processing software, such as ImageJ, a public domain Java-based program (http://rsb.info.nih.gov/ij/).

4. Notes

1. In oil seeds, like tobacco, the ^{1}H-NMR signal of "dry" seeds and of seeds during phase I water uptake (imbibition) is due to oil and water. In phase II, the water peak becomes prominent and what is detected is essentially water (see Fig. 1 of Manz et al. (3)). Separate MRI images of oil and water can be acquired using CSSI (Fig. 2) (18).
2. To obtain meaningful results from an in vivo ^{1}H-NMR microimaging experiment, images must be obtained for several seeds at the same stages. Representative typical images may then be selected for presentation.
3. When several phase III seeds are placed together in the capillary for ^{1}H-NMR microimaging, the subsequent computer separation can become problematic because of overlaps in the growing structures. This is not a problem with phase II seeds.

4. Small seeds require higher resolution and this means longer acquisition times. If acquisition times exceed several hours, the physiological changes in the seed need to be taken into account.

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Chapter 19

Tissue-Printing Methods for Localization of RNA and Proteins that Control Seed Dormancy and Germination

Wioletta E. Pluskota, Kent J. Bradford, and Hiro Nonogaki

Abstract

A number of genes and proteins are expressed in a tissue- or cell layer-specific manner. Spatial patterns of gene expression are critical to understanding gene function. Tissue printing provides a simple and rapid method to analyze localization of mRNA and protein at the tissue and cellular levels. This is especially convenient for gene expression analysis in hard tissues, such as seeds that are often difficult to section. Seed RNA or protein can be transferred onto a suitable membrane by printing the cut surface of a bisected seed. This method has been used successfully to determine mRNA and protein localization in seed research. The resolution of printed seed images and RNA and protein signals in tissue printing is sufficient to identify embryo- or endosperm-specific expression of various genes and proteins. In some cases, these studies have contributed to elucidating the spatial characteristics of hydrolytic enzymes putatively involved in the completion of germination and/or early postgerminative growth. By the same principle, tissue-printing methods could also be valuable for elucidating the spatial characteristics of genes/proteins that control the inception, maintenance, and termination of seed dormancy.

Key words: Tissue printing, Seed, mRNA, Protein, Localization, Gene expression, Dormancy, Germination

1. Introduction

Many genes involved in seed dormancy and germination have been identified (1–3). To understand the physiological roles of those genes and gene products and their regulatory mechanisms, it is necessary to characterize the timing and localization of gene expression in seeds. However, preparation of high-quality seed tissue sections for in situ hybridization is difficult and time consuming. Dissection of separate seed tissues, such as the embryo and endosperm, to examine tissue-specific gene expression by RNA gel blotting or RT-PCR is also laborious. Tissue printing (4) is a

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convenient alternative method to in situ hybridization or analysis of RNA samples extracted from various tissues.

Tissue-specific expression of gene family members appears to be a common feature of seed biology; seed tissue printing provides an excellent method to visualize and quantify localization of gene expression (5–7).

In this chapter, the value and application of seed RNA tissue printing are demonstrated by using the following examples (Subheading 3.5): (1) differentiation of the spatial expression patterns of *LeMAN1* and *LeMAN2* encoding endo-β-mannanases in tomato seed that share ~70% identity at the amino acid level (5) and (2) side-by-side comparison of a specific mRNA and the corresponding gene product (the protein) using the example of LeVA-P1, a binding domain of the vacuolar ATPase of tomato.

2. Materials

2.1. Seed Printing

1. Membranes: Positively charged nylon membrane (e.g., Amersham Hybond N$^+$, GE Healthcare, Piscataway, NJ) for RNA printing. Nitrocellulose membrane for protein printing (Optitran BA-S 85, Schleicher and Schuell, Keene, NH).
2. Double-edged razor blades.
3. Paper towels.
4. Powder-free gloves.
5. Parafilm.

2.2. RNA Detection (Northern)

2.2.1. Hybridization

1. Prehybridization buffer: 50% (v/v) deionized formamide, 2% (w/v) blocking reagent (Roche Molecular Biochemicals, Indianapolis, IN), 0.2% (w/v) SDS, 5× SSC. Store at −20°C. Prehybridization buffer should be equilibrated to the temperature used for hybridization before use.
2. Digoxigenin (DIG)-labeled antisense RNA probes.
3. Plastic bag (0.11 mm thick; KAPAK, Minneapolis, MN).
4. Plastic bag sealer (e.g., TEW Electric Heating Equipment Co., Ltd., Taipei, Taiwan).

2.2.2. Washing (After Hybridization)

1. Washing buffer 1: 2× SSC, 0.1% (w/v) SDS (prepare from 20× SSC and 10% SDS).
2. Washing buffer 2: 0.2× SSC, 0.1% (w/v) SDS (prepare from 20× SSC and 10% SDS).

2.2.3. DIG Antibody Reaction

1. Maleic acid buffer (10×): 1 M maleic acid, 1.5 M NaCl, 2 M NaOH. Heat is generated due to the exothermic reaction.

Cool down to room temperature before adjusting the pH to 7.5 with NaOH. Store at 4°C.

2. DIG antibody reaction buffer (buffer A): 1× maleic acid buffer containing 0.3% (v/v) Tween 20. Stir gently until Tween 20 is uniformly mixed. Store at 4°C.
3. DIG blocking buffer: Buffer A containing 5% (w/v) nonfat dry milk (stir at room temperature for 30 min).
4. Anti-DIG-alkaline phosphatase (AP) Fab fragments (0.75 U/μL; Roche Molecular Biochemicals). Store at 4°C.

2.2.4. Washing (After DIG Antibody Reaction)

Buffer A: 1× maleic acid buffer containing 0.3% (v/v) Tween 20.

2.2.5. Signal Detection (Alkaline Phosphatase Reaction)

Option A: Colorimetric detection

1. Equilibration buffer: 0.1 M Tris–HCl, 0.1 M NaCl, pH 9.5.
2. Tris–HCl buffer 1 M, pH 8.8.
3. $MgCl_2$ solution: 1 M $MgCl_2$.
4. Nitroblue tetrazolium chloride (NBT): 1 mg/mL in 0.2 M Tris–HCl, pH 8.8. Preparing fresh solution is recommended.
5. 5-Bromo-4-chloro-3-indolyl phosphate p-toluidine salt (BCIP): 10 mg/mL dimethylformamide (DMF). Preparing fresh solution is recommended.
6. Colorimetric enzyme reaction mixture: 0.18 M Tris–HCl, pH 8.8, 2 mM $MgCl_2$, 0.025 mg/mL BCIP, and 0.1 mg/mL NBT (prepare from 1 M Tris–HCl, pH 8.8, 1 M $MgCl_2$, NBT, and BCIP stock solutions).
7. Plastic bag (0.06 mm thick; KAPAKA, Minneapolis, MN).

Option B: Chemiluminescent detection

1. Equilibration buffer: 0.1 M Tris–HCl, 0.1 M NaCl, pH 9.5.
2. Chemiluminescent substrates (e.g., CDP-star Ready for Use, Tropix Inc, Bedford, MA or LumiPhos, Lumigen, Southfield, MI).

2.3. Protein Detection (Western)

1. Tris-buffered saline (TBS): 25 mM Tris, 0.15 M NaCl, adjust pH with HCl to 7.4.
2. TBS-Tween 20 (TBST): TBS containing 0.05% (v/v) Tween 20.
3. TBST blocking buffer: TBST containing 1% (w/v) bovine serum albumin (BSA).
4. Plastic bag (0.11 mm thick; KAPAK, Minneapolis, MN).
5. Primary antibodies (e.g., rabbit IgG specific to genes of interest).
6. TBST.
7. Secondary antibodies (e.g., anti-rabbit goat IgG conjugated with alkaline phosphatase).

3. Methods

In this section, methods for RNA detection using nonradioactive RNA probes and for protein detection using primary and secondary antibodies are described. The first step of seed printing is common for both RNA and protein detection, except for the nature of the membranes. Likewise, the final steps of both RNA and protein detection are similar in that the detection makes use of an enzyme (alkaline phosphatase) reaction for visualization of signals.

3.1. Seed Printing

1. Place an imbibed seed (see Note 1) on wet filter paper on a stage (e.g., a lid of plastic Petri dish can be used as a stage).
2. Cut a seed into halves by holding the seed vertically with forceps and using one side of a double-edged razor blade. (A double-edged razor blade can be wrapped with Kimwipes and cracked into halves with caution.) The thin blade of the razor gives a smoothly cut surface. Longitudinally bisected half seeds as well as transversally bisected seeds can be used for tissue printing. This procedure is best performed under a dissection microscope. To obtain a high-quality cut surface, each edge of the razor blade should not be used more than several times.
3. Place the half seed with its cut surface face down on a membrane. (A positively charged nylon membrane is used for RNA; nitrocellulose membrane is used for protein, see Subheading 2.)
4. Cover the membrane and half seed with parafilm and press hard with a thumb for ~15 s (see Note 2). Placing multiple layers of soft paper towels under the membrane is important to obtain a deep print of the seed (Fig. 1a). If the membrane is kept on a hard stage, the printed image will not be deep enough to visualize seed morphology. It is recommended that the resolution of printed images is examined under a dissection microscope before proceeding with hybridization.
5. Release pressure from the thumb gently and remove half seed with forceps. Avoid touching the membrane with the forceps to prevent extraneous marks on the prints and make sure that seed parts are not left on the membrane.
6. For tissue-printing RNA detection (Northern), cross-link RNA to the membrane using a UV cross-linker (see Note 3) and go to Subheading 3.2.

6′. For tissue-printing protein detection (Western), follow Subheading 3.3.

3.2. RNA Detection (Northern)

To analyze localization of gene expression, mRNA transferred to the membrane is hybridized with DIG-labeled antisense RNA probes, which are synthesized from cDNA by in vitro transcription

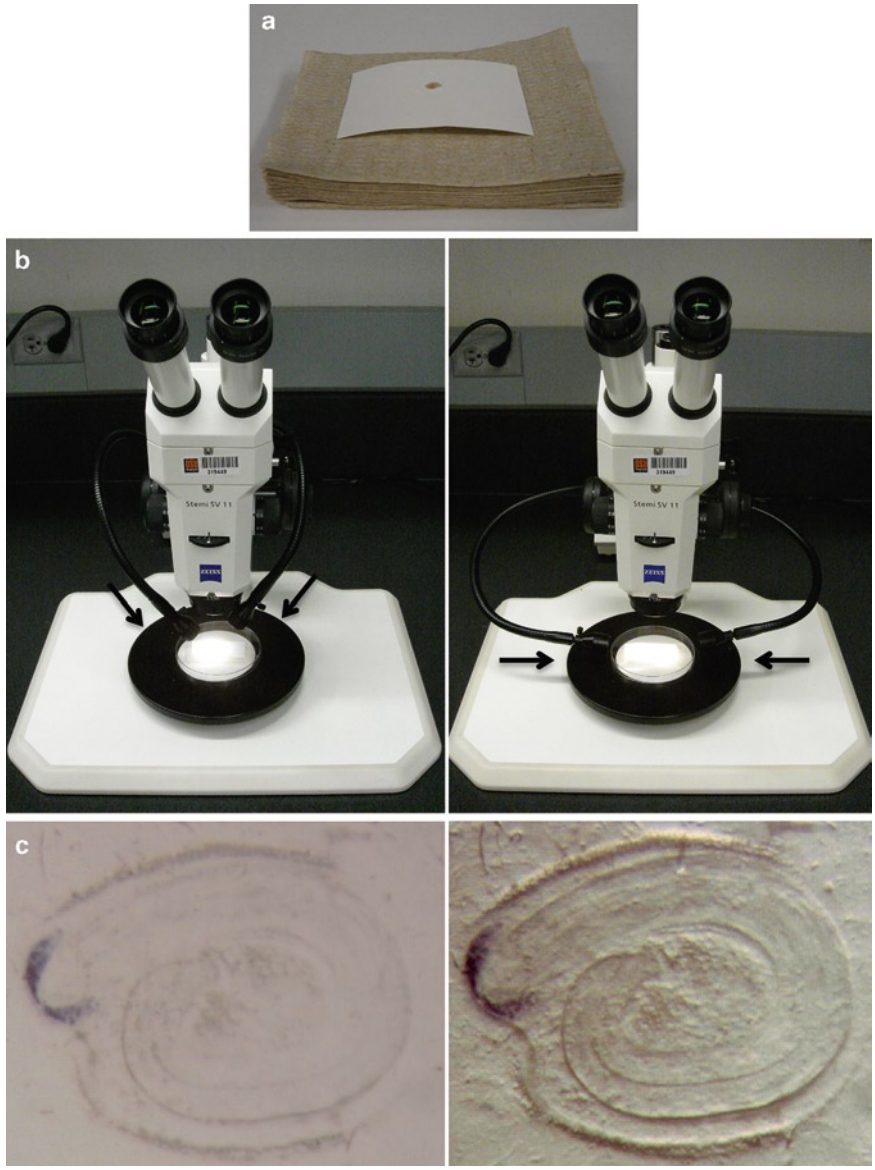

Fig. 1. Seed printing and image capturing. (**a**) A membrane placed on paper towels for tissue printing. (**b**) Examples of vertical (*left*) and horizontal (*right*) lighting to the tissue print membrane under a dissection microscope. (**c**) Tissue print images captured under vertical (*left*) and horizontal (*right*) lighting shown in (**b**). Note that the image captured under horizontal lighting has higher resolution of seed morphology (*right*) than the image under vertical lighting (*left*). Image: *LeMAN2* endo-β-mannanase mRNA expression in tomato seed.

with T7 or T3 RNA polymerase. DIG UTPs are incorporated into RNA molecules during synthesis. DIG molecules on the RNA probes are detected by anti-DIG antibody conjugated with alkaline phosphatase. The final detection of signals can be done using colorimetric or chemiluminescent substrates for alkaline phosphatase (see Note 4).

3.2.1. Hybridization

1. Incubate the membrane in prehybridization buffer equilibrated to an optimal temperature for 15 min. Temperature for hybridization depends on the probe used. For a specific probe of 1–2 kb length, 60°C in the presence of formamide works for hybridization and is stringent enough to avoid background. This step can be performed in a closed container.
2. Place the membrane between sealable plastic sheets (or bag). Add the appropriate volume of prehybridization buffer (0.1 mL/cm^2 membrane). Add the RNA probe (100 ng/mL) and incubate for 16 h (see Note 5).

3.2.2. Washing After Hybridization

1. Rinse the membrane briefly with washing buffer 1 and then wash with fresh washing buffer 1 equilibrated at 60°C once for 20 min.
2. Wash the membrane with washing buffer 2 at 60°C twice for 20 min (see Note 6).

3.2.3. DIG Antibody Reaction

1. Rinse the membrane with buffer A at room temperature.
2. Block the membrane with DIG blocking buffer at room temperature for 30 min using a shaker.
3. Add anti-DIG antibody (1:15,000) to the DIG blocking buffer and incubate at 25°C for 1 h.

3.2.4. Washing (After DIG Antibody Reaction)

1. Rinse the membrane briefly with buffer A.
2. Wash the membrane with buffer A at room temperature three times for 20 min using a shaker.

3.2.5. Signal Detection

1. Incubate the membrane in equilibration buffer at room temperature for 5 min to adjust pH environment (pH 9.5) for alkaline phosphatase.

Option A: Colorimetric detection

2. Place membrane in the colorimetric enzyme reaction mixture for 2 h to overnight with agitation (see Note 7).
3. Wash the membrane with water to remove the substrates and dry it at room temperature. The dried membranes can be kept at room temperature without losing signals or the original resolution of printed images.

Option B: Chemiluminescence detection

2′. Place the membrane with RNA side facing up on a plastic sheet.

3′. Apply chemiluminescent substrates for alkaline phosphatase (~10 μL/cm of membrane) and incubate at room temperature for 5 min.

4′. Cover the membrane with a plastic sheet (see Note 8).

5′. Expose to X-ray film for at least 15 min (CDPstar) or 30 min (LumiPhos).

3.3. Protein Detection (Western)

For protein detection, a combination of primary antibodies raised against a protein of interest and commercial secondary antibodies can be applied. The following is an example of protein detection using alkaline phosphatase-conjugated secondary antibodies.

3.3.1. Primary Antibody Reaction

1. Incubate membranes in TBST blocking buffer at room temperature for 30 min (see Note 9).
2. Add primary antibodies (e.g., 1:1,000) and incubate the membrane at 25°C for 1 h.

3.3.2. Washing (After Primary Antibody)

1. Rinse the membrane briefly with TBST.
2. Wash the membrane with TBST at room temperature three times for 10 min using a shaker.

3.3.3. Secondary Antibody Reaction

1. Add secondary antibody to TBST blocking buffer (e.g., 1:5,000) and incubate the membrane at 25°C for 1 h.

3.3.4. Washing (After Secondary Antibody)

1. Rinse the membrane briefly with TBST.
2. Wash the membrane with TBST at room temperature three times for 10 min using a shaker.

3.3.5. Signal Detection

See Subheading 3.2.5.

3.4. Capturing the Images

While tissue-printing signals and seed morphology are relatively stable on the membranes, it is recommended to take photographs of tissue prints with signals under a dissection microscope shortly after the signals are detected. To visualize the seed prints, it is helpful to give light laterally from both sides of a membrane rather than exposing light from above (Fig. 1b). The best resolution can be obtained by keeping membranes wet (or semidry) to visualize both the dark signal of RNA or proteins and the deep prints of seed tissues very clearly (Fig. 1c). Digital images can be analyzed by appropriate software for quantification of signal intensities.

3.5. Application of the Techniques for Understanding Seed Biology

1. Differentiating the spatial expression patterns of related genes: Tissue-specific expression of gene family members is a common feature of seed biology (8–11). As an example, seed RNA tissue printing was used to differentiate the spatial expression patterns of *LeMAN1* and *LeMAN2* encoding endo-β-mannanases in tomato seed that share ~70% identity at the amino acid level ((5); Fig. 2). The germination-specific gene *LeMAN2* exhibits expression confined to the micropylar region of the endosperm

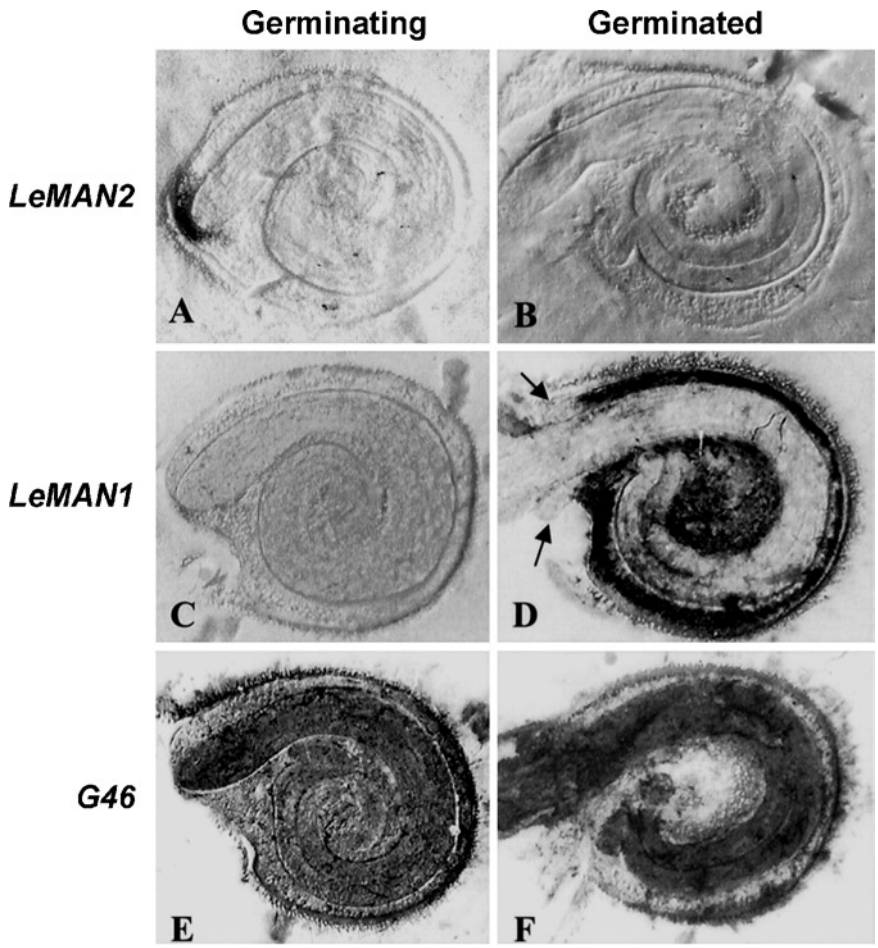

Fig. 2. Tissue printing and hybridization of germinating (24-h imbibed) and germinated tomato seeds. Prints were hybridized with antisense riboprobes for *LeMAN2* (**a**, **b**), *LeMAN1* (**c**, **d**), and *G46* encoding a constitutively expressed ribosomal protein used as a control (**e**, **f**). The *arrows* in (**d**) indicate the remaining endosperm cap tissue in the germinated seeds, which do not hybridize with the *LeMAN1* probe. From ref. 5; used with permission of the American Society of Plant Biologists.

(the endosperm cap) before radicle emergence, whereas *LeMAN1* is expressed in the rest of the endosperm specifically after germination (Fig. 2). The signal from a constitutively expressed gene provides a good control for the efficiency of RNA transfer to the membrane and can be used for normalization of the signal intensity from individual seeds for quantification (Fig. 2e, f).

2. Side-by-side comparison of a specific mRNA and the corresponding gene product (protein): Another advantage of seed tissue printing is that multiple seed samples can be printed on a membrane and the signals from individual seeds for a specific mRNA or protein can be compared side by side across treatments or conditions (12). In addition, both mRNA and proteins can

be detected using tissue prints. For example, a print of one half of a bisected seed can be exposed to an RNA probe and the other half of the same seed (mirror image print) can be reacted with antibodies specific to the gene product. Figure 3a demonstrates an example of detection of mRNA and protein in individual seeds in the case of LeVA-P1, a binding domain of the vacuolar ATPase of tomato (A. Mella et al., unpublished results). In this case, two different reactions (RNA hybridization and antibody binding) were performed on prints of the opposing halves from individual seeds. The results obtained by tissue printing were then quantified based on the digital image intensities, which showed a good correlation between mRNA and protein accumulation in individual seeds (Fig. 3b). Alternatively, the same print can be stripped after hybridization

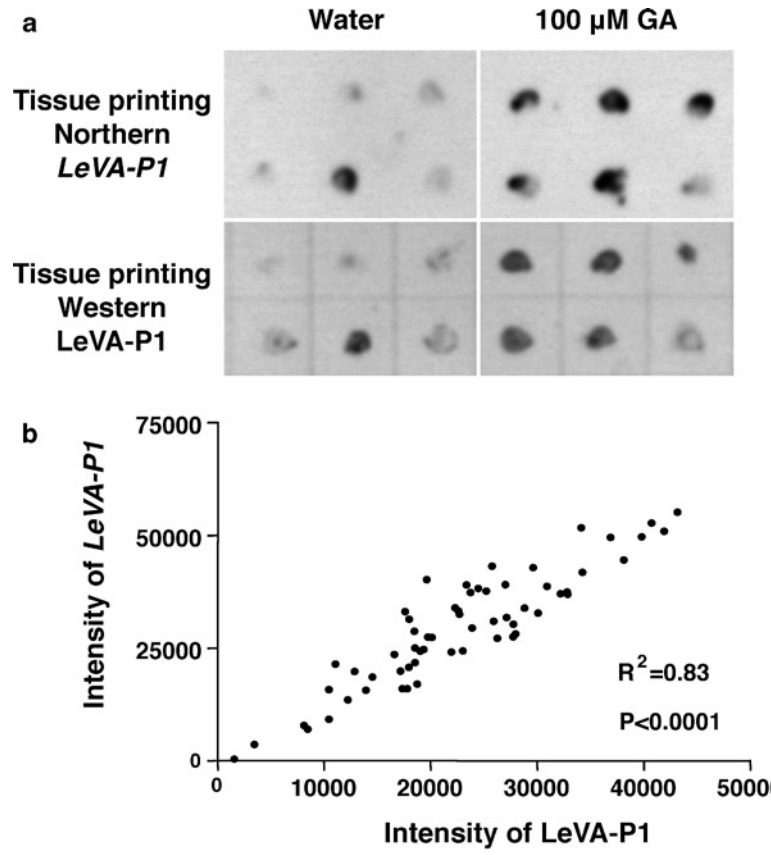

Fig. 3. Northern and Western tissue prints. The seeds were sliced in the middle and both halves printed in mirror membranes. One membrane was developed as Northern (*LeVA-P1*, bean vacuolar ATPase) and the other as Western (antibody against LeVA-P1). (**a**) Mirror images of seeds treated with water or 100 µM gibberellin (GA_{4+7}). (**b**) Regression of the digitized measurements of the mRNA and protein intensities of the mirror images of individual seeds (intensities in arbitrary units). Previously unpublished data of A. Mella et al.

with one probe (e.g., a probe to a gene of interest) and rehybridized with another probe (e.g., a probe to a constitutively expressed gene).

4. Notes

1. Seeds must be imbibed sufficiently to transfer RNA or proteins efficiently to a membrane. Therefore, very early stages of imbibition (several hours) may not be suitable for tissue-printing analyses.
2. When seed printing is attempted for a long time (>15 s), the prints tend to result in fuzzy images probably due to small movement during imprinting.
3. To avoid the degradation of mRNA, do not keep membranes with prints for a long time before hybridization. It is better to use freshly prepared membranes for hybridization.
4. Detection sensitivity is high when chemiluminescent substrates are used and signals are detected on an X-ray film. However, seed morphology is not visualized in an X-ray film.
5. Add a probe directly to the prehybridization buffer without touching membranes or the inner sides of plastic sheets.
6. High stringency (high temperatures and presence of formamide) of washing gives highly specific signals with very low background in tissue prints.
7. Duration of the final enzyme reaction for signal detection varies depending on the amount of transcripts expressed or proteins synthesized and the concentration of the specific probes used for hybridization.
8. When CDPstar is used as a chemiluminescent substrate, gently squeeze out excessive substrate solution. This reduces background in an X-ray film.
9. There are some occasions, where 1.5% (v/v) Tween 20 works better than 1% BSA in the TBST blocking buffer for protein detection (Western) (J. Buitink, personal communication).

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Part VII

Analyses of Reactive Oxygen Species, Protein Damage and Repair

Chapter 20

Analyses of Reactive Oxygen Species and Antioxidants in Relation to Seed Longevity and Germination

Christophe Bailly and Ilse Kranner

Abstract

Evidence is emerging that reactive oxygen species (ROS) and antioxidants, together with plant hormones and other reactive species, such as reactive nitrogen species, are part of signalling networks pertinent to plant stress responses, cell division, and cell death. Consequently, they play pivotal roles in the regulation of seed development and maturation, germination and dormancy, seedling establishment, and seed ageing. Importantly, ROS, although essentially required at low concentrations, must be kept under stringent control by antioxidants. If the balance between pro- and antioxidative processes is lost and ROS production prevails, oxidative stress is the result, which can induce cell death and ultimately seed death. This chapter offers a variety of protocols for the determination of ROS, antioxidants, and stress markers aimed at enabling the reader to quantify these compounds. Protocols are also described to visualize ROS and localize the sites of ROS production, hoping to stimulate more research into ROS signalling and antioxidant control in key physiological and biochemical processes in seeds.

Key words: Antioxidants, Ascorbate, Seed germination, Glutathione, Hydrogen peroxide, Reactive oxygen species, Superoxide, Tocopherol

1. Introduction

Reactive oxygen species (ROS) include the superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($^{\bullet}OH$) and are continuously produced in plants as by-products of various metabolic pathways. In seeds, ROS are involved in deterioration during storage (1). However, evidence is now accumulating that ROS also play important roles in signalling associated with germination and dormancy alleviation (1). In order to obtain a comprehensive view of ROS metabolism in seeds, both the changes in ROS production and their regulation by antioxidants must be considered. ROS can be scavenged through the action of detoxifying

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enzymatic cascades that include enzymes, such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), or glutathione reductase (GR), and by low-molecular-weight antioxidants among which ascorbate (AA), tocopherol, and glutathione (GSH) are the most abundant (2). Phenolic compounds often present in seed coats also have strong antioxidant properties, but are not considered in this chapter. We outline biochemical methods for assessing the balance between ROS and antioxidant mechanisms in seeds as well as techniques for determining ROS production sites in situ. Such methods can be complemented by classical molecular biology tools (not described here) to study the expression of genes regulated by ROS (3). Moreover, ROS also react with essential biomolecules, including lipids, proteins, and nucleic acids, which can lead to changes in cellular signalling or to damage if ROS scavenging pathways succumb under increasing stress and ROS production prevails. For example, proteins are very susceptible to oxidation, which can result in protein carbonylation. Methods to study protein carbonylation are described in Chapter 14. Lipids are also one of the major targets of ROS and estimation of their peroxidation described here can be used as a marker of oxidative stress.

2. Materials

2.1. Determination of ROS

2.1.1. Hydrogen Peroxide Determination: 3-Methyl-2-benzothiazolinone hydrazone Method

1. Perchloric acid 0.2N.
2. Potassium hydroxide 4N.
3. 3-Dimethylaminobenzoic acid (DMAB): 12 mM in 0.375 M phosphate buffer, pH 6.5. DMAB must be first dissolved in ~200 µL ethanol.
4. 3-Methyl-2-benzothiazolinone hydrazone (MBTH), 1.3 mM.
5. Horseradish peroxidase (HRP) type VI: Stock solution of 12.5 U/mL.
6. Spectrophotometer and appropriate consumables, such as cuvettes.

2.1.2. Hydrogen Peroxide Determination: Luminol Method

1. Trichloroacetic acid 5% (w/v).
2. Ammonium hydroxide, 0.2 M.
3. Ion exchange resin Dowex 1X8 (Cl^- form).
4. Luminol, 0.05 mM.
5. Potassium ferricyanide, 0.05 mM.
6. Hydrogen peroxide solutions: 1 µM to 1 mM in TCA 5% (for preparing standard curve). Prepare fresh daily.

7. Luminometer and appropriate consumables, such as glass test tubes.
8. Spectrophotometer and appropriate consumables, such as cuvettes.

2.1.3. Analysis of Extracellular Hydrogen Peroxide Production: Xylenol Orange Assay

1. Reagent A: 25 mM $FeSO_4$, 25 mM $(NH_4)_2SO_4$, and 2.5 M H_2SO_4.
2. Reagent B: 125 μM xylenol orange and 100 mM sorbitol.
3. The "working reagent": 0.1 mL of reagent A and 10 mL of reagent B. Incubate for 15 min (or until no further colour change occurs) before use.
4. Commercially available (Sigma-Aldrich) stock solutions of CAT and H_2O_2.
5. Spectrophotometer and appropriate consumables, such as cuvettes.

2.1.4. Superoxide Determination: Hydroxylamine Method

1. Sodium phosphate buffer: 50 mM, pH 7.8.
2. 1 mM hydroxylamine hydrochloride in 50 mM sodium phosphate buffer, pH 7.8.
3. Sulphanilamide, 17 mM.
4. 2-Naphtylamine, 7 mM.
5. Solutions of sodium nitrite in sodium phosphate buffer (pH 7.8, 50 mM) ranging from 0.1 μM to 1 mM for standard curve.
6. Spectrophotometer and appropriate consumables, such as cuvettes.

2.1.5. Analysis of Extracellular Superoxide Production: Epinephrine and XTT Assays

1. Epinephrine: 1 mM, pH 7.0.
2. Sodium 3′[-1-(phenylamino-carbonyl-carbon)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene-sulphonic acid hydrate (XTT): 0.5 mM.
3. NADH: 0.2 mM. Prepare fresh.
4. SOD: 250 U/mL.
5. Spectrophotometer and appropriate consumables, such as cuvettes.

2.2. ROS Localization

All chemicals used should be of analytical grade quality. All aqueous solutions should be made with double-distilled H_2O.

2.2.1. Hydrogen Peroxide Localization with Cerium Chloride

1. Cerium chloride: 5 mM $CeCl_3$ in 50 mM MOPS buffer, pH 7.2.
2. Glutaraldehyde: 6% (v/v) glutaraldehyde in 25 mM sodium phosphate buffer, pH 7.2.

3. Osmium tetroxide: 1% (v/v) OsO_4 in 25 mM sodium phosphate buffer, pH 7.2.
4. Graded series of ethanol: 25, 50, 75, 95, and 100% (v/v).
5. Propylene oxide.
6. Araldite®.
7. Standard equipment for transmission electron microscopy, including ultra-microtome.

2.2.2. Tissue Localization of Hydrogen Peroxide with TMB

1. McIlvaine buffer, pH 6.0: For 100 mL, mix 36.85 mL of 0.1 M citric acid with 63.15 ml of 0.2 M Na_2HPO_4.
2. 3,5,3′,5′-tetramethylbenzidine (TMB): 1% (w/v) TMB in McIlvaine buffer.

2.2.3. Hydrogen Peroxide Localization with Amplex® Red

1. Stock solution of Amplex® Red: 20 mM in dimethyl sulfoxide (DMSO).
2. Potassium phosphate buffer: 20 mM, pH 6.0.
3. HRP type VI: Prepare a stock solution of 10 U/mL.
4. Standard equipment for fluorescence microscopy or confocal laser scanning microscope.

2.2.4. Superoxide Localization with NBT

1. Nitroblue tetrazolium chloride (NBT): 6 mM NBT in 10 mM Tris–HCl buffer, pH 7.4.
2. Standard equipment for light microscopy.

2.2.5. ROS Localization with DCFH-DA

1. Stock solution of 5-(and-6)-chloromethyl-2′,7′-dichlorofluorescein diacetate (DCFH-DA): 25 mM DCFH-DA in acetone.
2. Working solution of DCFH-DA: 100 µM DCFH-DA in 20 mM potassium phosphate buffer, pH 6.0.
3. Standard equipment for fluorescence microscopy or confocal laser scanning microscope.

2.3. Antioxidant Systems

2.3.1. Enzyme Extraction

1. Extraction buffer: 0.1 M potassium phosphate buffer, pH 7.8, containing 2 mM dithiothreitol (DDT), 0.1 mM ethylenediaminetetraacetic acid (EDTA), and 1.25 mM polyethylene glycol 4000 (PEG 4000).
2. Polyvinylpolypyrrolidone (PVPP).
3. Sephadex G25 column (i.e., disposable PD10 desalting columns, GE Healthcare).

2.3.2. Enzyme Analysis: CAT

1. Potassium phosphate buffer: 50 mM potassium phosphate, pH 7.0. Ensure that the solution has equilibrated to room temperature before starting the measurements.
2. Hydrogen peroxide: 37.5 mM. Prepare fresh daily.

3. Spectrophotometer and appropriate consumables, such as cuvettes.

2.3.3. Enzyme Analysis: Superoxide Dismutase

1. Potassium phosphate buffer: 0.1 M potassium phosphate, pH 7.8. Ensure that the solution is in equilibrium with room temperature before starting measurements.
2. Riboflavin, 15.6 μM.
3. Methionine, 0.15 M.
4. NBT, 756 μM.
5. Glass test tubes, temperature-controlled water bath, fluorescent lamp (500 W min) (see Note 1).
6. Spectrophotometer and appropriate consumables, such as cuvettes.

2.3.4. Enzyme Analysis: Glutathione Reductase

1. Potassium phosphate buffer: 0.1 M potassium phosphate, pH 7.8. Ensure that the solution is at room temperature before starting measurements.
2. Glutathione disulphide (GSSG), 160 mM.
3. NADPH, 8 mM.
4. $MgCl_2$, 24 mM.
5. Spectrophotometer and appropriate consumables, such as cuvettes.

2.3.5. Enzyme Analysis: Ascorbate Peroxidase

1. Potassium phosphate buffer: 50 mM potassium phosphate, pH 7.0. Ensure that the solution is at room temperature before starting measurements.
2. Ascorbate, 5 mM.
3. Hydrogen peroxide 20 mM. Prepare fresh daily.
4. Spectrophotometer and appropriate consumables, such as cuvettes.

2.3.6. Analysis of Lipid-Soluble Antioxidants: Tocopherols and Carotenoids

All chemicals used should be of analytical grade quality.

1. Extraction medium: DMSO, ice-cold ethanol, acetone, or acetone containing 0.1% N-ethyldiisopropylamine (see Note 2).
2. $CaCO_3$ or $MgCO_3$ (carotenoids only).
3. HPLC solvent for tocopherols: Methanol, HPLC grade.
4. HPLC solvents for carotenoids: *Solvent A:* acetonitrile: water: methanol = 100:10:5 (v/v/v); *solvent B:* acetone:ethyl acetate = 2:1 (v/v).
5. Pigments for preparation of standards: These are available from Sigma-Aldrich and DHI – Water & Environment (Denmark).
6. Freeze drier (optional; see Note 3).

7. For freeze-dried samples: Laboratory ball mill with Teflon grinding vessels and agate balls (e.g. Retsch MM200 or Braun Dismembrator). Alternatively, mortar and pestle; both grinding techniques (see Note 4) require liquid nitrogen.
8. HPLC system consisting of two high-pressure pumps (for carotenoids) or one pump (for tocopherols), a sample injector with a 20-µL dosing loop, or (optional) an auto-sampler preferably with cooling option; RP-18 column (250×4.6 mm i.d., 5-µm particle size); for carotenoids, a UV/Vis variable wavelength detector or photodiode array detector (optional) and for tocopherols, a fluorescence detector (see Note 5) and data collection and integration software.

2.3.7. Analysis of Water-Soluble Antioxidants and Their Redox State: GSH and GSSG

All chemicals used should be of analytical grade quality; all aqueous solutions should be made up with double-distilled H_2O.

1. Extraction medium: 0.1 M HCl containing 0.5% Nonidet or Triton X-100 (the addition of detergents is optional, but can enhance the extraction efficiency).
2. 3 mM DL-DTT. Prepare fresh daily.
3. PVPP.
4. 2-(*N*-cyclohexylamino)ethanesulfonic acid (CHES) buffer: 200 mM CHES buffer containing 1 mM EDTA (optional, to remove any redox-active metals that may be present). Adjust to pH 9.3 with 10 M NaOH. Note that the mixture of 400 µL sample extract and 600 µL CHES buffer must have a pH between 8 and 8.3, which is the optimal pH range at which monobromobimane (mBBr) binds to thiols. If the pH of the mixture lies outside of this range, adjust the pH of the CHES buffer accordingly.
5. 50 mM *N*-ethylmaleimide (NEM) dissolved in 2-propanol (see Note 6).
6. 15 mM mBBr dissolved in acetonitrile (see Note 6).
7. 0.25% (v/v) methanesulphonic acid (MSA).
8. Thiol standards: Stock solutions of 1 mM GSH, 0.1 mM cysteine, 0.1 mM γ-glutamyl-cysteine, and 0.1 mM cysteinyl-glycine prepared in 0.1 M HCl. In addition to GSH, some plant families contain GSH homologues. For example, the Poaceae and Leguminosae, respectively, contain hydroxylmethyl glutathione (γ-glutamyl-cysteinyl-serine) and homoglutathione (γ-glutamyl-cysteinyl-β-alanine). Standards of GSH homologues can be obtained from Bachem (St Helens, Merseyside, UK). Aliquots of standard solutions can be shock-frozen in liquid nitrogen and stored at –80°C, but thaw only once and discard the remainder.
9. HPLC solvent A: 0.25% (v/v) acetic acid. Make up with double-distilled water, adjust to pH 3.9 with 10 M NaOH, and filter

through a 0.2-μm Millipore filter before use. Solvent B: HPLC-grade methanol.

10. HPLC system consisting of two high-pressure pumps (see Note 7), a sample injector with a 20-μL dosing loop, or (optional) an auto-sampler with cooling option; an RP-18 column (250 × 4.6 mm i.d., 5-μm particle size; for example, an HiQsil C18V column from KyaTech, Japan); a fluorescence detector (see Note 5); and data collection and integration software.

2.3.8. Analysis of Water-Soluble Antioxidants and Their Redox State: Ascorbate and Dehydroascorbate

All chemicals used should be of analytical grade quality; all aqueous solutions should be made with double-distilled H_2O.

1. Extraction medium: 1 mL 1.5% (w/v) metaphosphoric acid containing 1 mM EDTA disodium salt dihydrate.
2. 1,2-phenylenediamine dichloride (1 mg per 1 mL water).
3. 0.2 M aqueous Tris.
4. HPLC solvent: Methanol:water (1:3; v/v; HPLC grade) containing 1 mM hexadecyltrimethylammonium bromide and 0.05% (w/v) sodium dihydrogen phosphate monohydrate. Adjust pH to 3.6 by the addition of 85% (w/v) *ortho*-phosphoric acid.
5. HPLC system consisting of a pump, a sample injector with a 20-μL dosing loop, or (optional) an auto-sampler with cooling option; RP-18 column (250 × 4.6 mm i.d., 5-μm particle size); photodiode array detector (alternatively, the analyte can be injected twice using different wavelengths for data collection); and data collection and integration software.

2.4. Markers of Oxidative Stress

2.4.1. Marker of Lipid Peroxidation: Malondialdehyde

1. Thiobarbituric acid (TBA)–trichloroacetic acid (TCA) solution: 0.5% (w/v)–20% (w/v).
2. Temperature-controlled water bath, spectrophotometer, and appropriate consumables, such as cuvettes.

2.4.2. Marker of Lipid Peroxidation: Conjugated Dienes

1. Chloroform, ethanol, gaseous nitrogen.
2. EDTA: 5 mM EDTA stabilized with NaCl 1% (w/v).
3. Spectrophotometer and appropriate consumables, such as cuvettes.

3. Methods

We first offer several methods for the assessment of intracellular ROS so that the users can choose the most appropriate one for their experimental system or according to the availability of equipment

and chemicals. Many different methods exist to assess the contents of H_2O_2 and $O_2^{\bullet-}$ in plant tissues, but the ones we outline here have been successfully used in seeds of various plant species and are reliable. Nonetheless, it must be noted that accurate ROS measurements require careful attention, caution, and practice because of their reactivity with other compounds, which may alter their accurate estimation (4). The methods described here are either spectophotometric for H_2O_2 (the DMAB-MBTH method; 5) and $O_2^{\bullet-}$ [the hydroxylamine method; 6) or based on light emission for H_2O_2 [the chemiluminescence method, 7). Moreover, ROS can also be produced extracellularly by cell wall-bound enzymes and released into the apoplast, where they play important roles in cell-to-cell signalling. Evidence is emerging that the relatively long-lived $O_2^{\bullet-}$ and H_2O_2 are likely candidate ROS involved in extracellular signalling. Methods for the assessment of extracellular H_2O_2 production (8) and $O_2^{\bullet-}$ (9–11) are included. Attention is also given to the localization of ROS either at the tissue or at the cellular level. This can be particularly useful for studying the involvement of ROS in germination or in other seed-specific processes, such as endosperm weakening. Specific methods for localization of H_2O_2 (12–14) and $O_2^{\bullet-}$ (15) are provided, but we also describe a method for general ROS localization with DCFHDA (16), a compound that can be oxidized by various oxidative species (17) and may be used to give an indication of oxidative stress.

The next section of this chapter describes methods for the analyses of antioxidant enzymes and low-molecular-weight antioxidants that can scavenge ROS individually or in cooperation, where they are intricately involved in complex ROS-processing and ROS-scavenging biochemical cascades. Assays for enzyme activity are included for the ROS-processing enzymes, SOD (18), CAT (19), GR (20), and APX (21). The regulation of most of these enzymes in seeds is mainly transcriptional, and the estimation of activities can also give insights into gene expression (22). For low-molecular-weight antioxidants, particular attention is given to the determination of the antioxidant redox state, concentrating on the redox couples of the most abundant intracellular water-soluble antioxidants, GSH/GSSG (23, 24) and AA/DHAA (25). In addition, protocols for the determination of lipid-soluble antioxidants (tocopherols and carotenoids) are included (26). Most carotenoids have antioxidant properties; they can quench ROS or dissipate excess light energy, but their importance goes beyond photosynthesis and participating in oxidative stress responses. For example, they are required for abscisic acid synthesis. Hence, this protocol may be useful for studies into the hormonal control of seed longevity, dormancy, and germination. In addition, some seeds may also contain chlorophyll or chlorophyllids in their cotyledons or the seed coat which can be co-identified with carotenoids. Please note that the

procedures for seed handling (freeze drying, storage, and grinding) are only described once in the protocol for extraction of lipid-soluble antioxidants, but also apply to the protocols for water-soluble antioxidants.

Finally, methods for the analyses of markers of oxidative stress are given with a focus on lipid peroxidation, as lipids are among the major targets of ROS attack in cells (determination of protein oxidation is dealt with in Chapter 14). We provide methods for the detection of lipid peroxidation by-products, such as malondialdehyde (MDA) (27) and conjugated dienes (28).

We generally recommend using all chemicals of the highest purity available, in particular for HPLC analyses. For the latter, all aqueous solutions must be made up with double-distilled water. Unless indicated otherwise, all required chemicals can be purchased from Sigma-Aldrich or Fisher.

3.1. Determination of ROS

3.1.1. Hydrogen Peroxide Determination: DMAB/MBTH Method

1. Seeds (see Note 8) are ground in an ice-cold mortar and homogenized with 0.2 M perchloric acid (approximately 0.1 g dry weight (DW)/mL).
2. After 15 min of centrifugation at 13,000 × *g* at 4°C, the resulting supernatant is neutralized to pH 7.5 with 4 M KOH (see Note 9) and then centrifuged at 1,000 × *g* for 3 min at the same temperature.
3. The supernatant is immediately used for spectrophotometric determination of H_2O_2 at 590 nm using a peroxidase-based assay. Mix 950 µL H_2O, 400 µL of 12 mM DMAB, and 80 µL of 1.3 mM MBTH in a spectrophotometer cuvette and then make the blank at 590 nm. Fifty microlitres of the extract are then added, the absorbance of the mixture is noted, and the reaction is initiated by the addition of 20 µL of HRP. The increase in absorbance at 590 nm is monitored after 5–10 min at 25°C and compared with the absorbance obtained with known amounts of H_2O_2 (to be introduced in the reaction mixture instead of the extract). The relationship between absorbance and H_2O_2 concentration should be linear between 1 µM and 0.5 mM and allow calculation of the amount of H_2O_2.

3.1.2. Hydrogen Peroxide Determination: Luminol Method

1. Grind seeds in liquid nitrogen and homogenize in TCA (0.1 g DW/mL TCA) and then centrifuge for 15 min at 14,000 × *g* and 4°C.
2. Mix 0.5 mL of the supernatant with 0.5 ml of TCA and 0.5 g of Dowex previously equilibrated for 5 min with TCA. After 5 min of gentle agitation, centrifuge the mixture for 15 min at 14,000 × *g* and 4°C.
3. Mix 100 µL of the supernatant with 700 µL of NaOH and 100 µL of luminol in a glass test tube. Record light emission using a luminometer for 10 s and then inject 50 µL FeCN into

the tube. Light emissions of seed samples are typically measured during 60 s and compared to those obtained using solutions of various concentrations of H_2O_2 in TCA to produce a standard curve.

3.1.3. Analysis of Extracellular Hydrogen Peroxide Production: Xylenol Orange Assay

1. Shake seeds in distilled water for 30 min (or longer, depending on the experiment and species used). If isolated embryonic axes are used, note that axis excision induces an oxidative burst that may include H_2O_2. The number of seeds and the water volume need to be altered for each plant species. For pea-sized seeds, ten seeds incubated in 15 ml of distilled water may produce conveniently measurable H_2O_2 concentrations. For isolated axes, five axes incubated in 5 ml may provide suitable readings.
2. After 30 min, add a 0.6-mL aliquot of the leachate to 3 mL of working reagent and incubate for 15–20 min at room temperature. To measure H_2O_2 spectrophotometrically, record the absorbance of the resulting solution against a blank (3 mL of working reagent and 0.6 mL of water) at A_{560} and calculate the concentration using a calibration curve.
3. To test the specificity of the assay, H_2O_2 in seed leachates should be measured in the presence of 250 U/mL of CAT. Also include CAT in blanks.

3.1.4. Superoxide Determination: Hydroxylamine Method

1. Grind seeds (~0.2 g DW) in 4 mL of sodium phosphate buffer (pH 7.8, 50 mM) at 4°C. Centrifuge the extract at 16,000 × *g* for 15 min and use the resulting supernatant for $O_2^{\bullet-}$ determination.
2. Incubate 1 mL of the supernatant at 25°C for 30 min in the presence of 1 mM hydroxylamine hydrochloride. Then, incubate 0.5 mL of this reaction mixture with 0.5 mL of 17 mM sulphanilamide and 0.5 mL of 7 mM 2-naphtylamine at 25°C for 30 min. Measure the absorbance at 540 nm after centrifugation at 13,000 × *g* for 10 min, and calculate $O_2^{\bullet-}$ concentrations using a calibration curve obtained with solutions of various concentrations of sodium nitrite as described above.

3.1.5. Analysis of Extracellular Superoxide Production

1. For the epinephrine assay, shake seeds at 45 revolutions per minute in 15 mL of 1 mM epinephrine, pH 7.0 (if required, adjust pH with a pinch of $NaHCO_3$), for 30 min at 25°C. For seed numbers and incubation volumes, follow the advice given in Subheading 3.1.3.
2. Using an aliquot of the incubation solution, record the $O_2^{\bullet-}$-dependent oxidation of epinephrine to adrenochrome at A_{490} using an extinction coefficient (ε) of 4.02/mM/cm (9).
3. For the XTT assay, incubate seeds in 15 mL of 0.5 mM XTT for 30 min at 25°C. In this assay, XTT is reduced by $O_2^{\bullet-}$ to

a soluble formazan. Measure an aliquot of the incubation solution at A_{470} at pH 7.0 and then calculate $O_2^{\bullet -}$ concentrations ($\varepsilon = 21.6/\text{mM/cm}$). If activities are low, reductants, such as 0.2 mM NADH, can be added to stimulate $O_2^{\bullet -}$ production.

4. To test the specificity of the assays, measure $O_2^{\bullet -}$ in the leachates in the presence of 250 units/mL SOD (see Note 10).

3.2. ROS Localization

3.2.1. Intracellular Hydrogen Peroxide Localization with Cerium Chloride

1. Imbibe seed sections (approximately 5 mm^3) in 5 mM $CeCl_3$ under vacuum until they are fully infiltrated.
2. Immediately fix $CeCl_3$-treated sections and control sections (without staining) in 6% glutaraldehyde in 25 mM sodium phosphate buffer (pH 7.2) for 24 h at room temperature and then wash in the same buffer and postfix in 1% osmium tetroxide in 25 mM sodium phosphate buffer (pH 7.2) for 18 h at room temperature.
3. After several washes (3–4) in double-distilled water, progressively dehydrate the tissues in ethanol (successively 1 h each in 25, 50, 75, and 95% ethanol and three times 1 h each in 100% ethanol) and soak in propylene oxide/ethanol (v/v) for 1 h and three times (1 h each) in pure propylene oxide. Then, soak samples for 18 h in propylene oxide/Araldite® (3v/1v), 12 h in propylene oxide/Araldite® (v/v), 18 h in propylene oxide/Araldite® (1v/3v), and 4 h in pure Araldite®. Embed samples in Araldite® at 60°C for 48 h. Section the blocks with a glass knife at 120 nm using a microtome, stain the sections with lead citrate and 2% uranyl acetate, and view with a transmission electron microscope (TEM). Hydrogen peroxide appears as black spots, which correspond to water-insoluble cerium perhydroxide precipitates formed after the reaction of $CeCl_3$ with endogenous H_2O_2 (Fig. 1a).

3.2.2. Tissue Localization of Hydrogen Peroxide with TMB

1. Incubate seeds at room temperature in the dark for 15 min in 1% TMB in 1/10 McIlvaine buffer.
2. Blue staining develops at the sites of H_2O_2 production.

3.2.3. Hydrogen Peroxide Localization with Amplex® Red

1. Prepare 5 mL working solution of Amplex® Red by mixing 4.85 mL of potassium phosphate buffer, 50 µL Amplex® Red stock solution, and 100 µL HRP (10 units/mL).
2. Incubate seeds or hand-cut seed sections at room temperature in the dark for 15 min in the working solution.
3. Rinse samples three times for 5 min with potassium phosphate buffer.
4. View samples with a fluorescence microscope or confocal laser scanning microscope (excitation: 530 nm, emission: 590 nm).

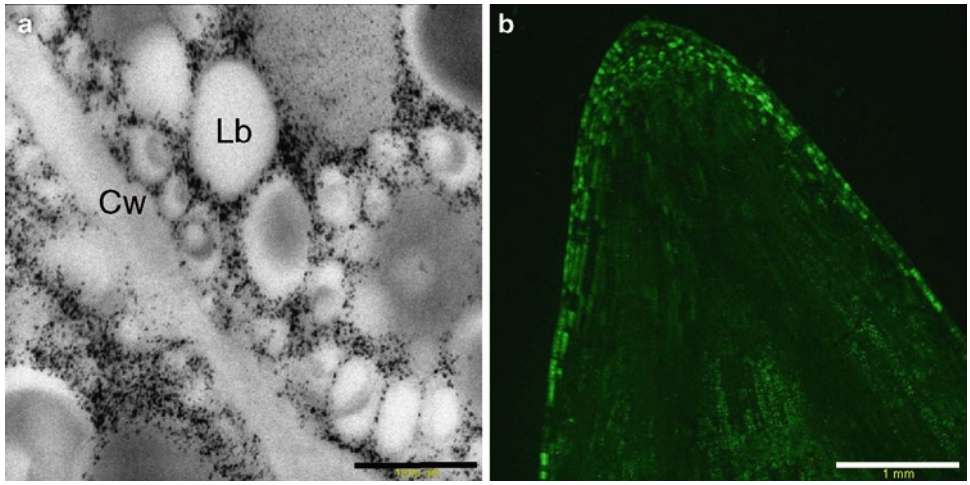

Fig. 1. Localization of ROS in embryo tissues. (**a**) Sub-cellular localization of H_2O_2 in sunflower embryonic axes viewed by TEM after $CeCl_3$ staining. H_2O_2 production is visualized as black spots corresponding to electron-dense cerium perhydroxide precipitates. Cw, cell wall; Lb, lipid body. Bar = 1 μm. (**b**) Fluorescence imaging of ROS production sites in embryonic axes of sunflower seeds after treatment with DCFH-DA, visualized by confocal laser scanning microscopy. The intensity of fluorescence is proportional to ROS accumulation. Bar = 1 mm.

3.2.4. Superoxide Localization with NBT

1. Incubate seed material in 6 mM NBT in 10 mM Tris–HCl buffer (pH 7.4) at room temperature for 15–30 min.
2. Rinse with distilled water; $O_2^{\bullet -}$ is visualized as deposits of dark blue insoluble formazan compounds that can be seen at the tissue level. Intracellular deposits may be viewed using photon microscopy.

3.2.5. ROS Localization with DCFH-DA

1. Incubate seeds or hand-cut seed sections at room temperature in the dark for 15 min with DCFH-DA.
2. Rinse samples three times with buffer for 5 min each.
3. View sections or whole seeds with a fluorescence microscope or confocal laser scanning microscope (excitation: 488 nm, emission: 525 nm) (Fig. 1b).

3.3. Antioxidant Systems

3.3.1. Enzyme Extraction

1. Carry out all extraction steps at 4°C. Homogenize seeds (see Note 11) in a chilled mortar with extraction buffer in the presence of insoluble 20% PVPP (w/v). Vortex the homogenate for 1 min and centrifuge at 11,000 × *g* for 15 min.
2. Filter the supernatant through Miracloth and desalt on a Sephadex G25 column using 0.1 M potassium phosphate buffer (pH 7.8). The obtained extract is used for determination of enzyme activities. It can be stored at −30°C, but avoid numerous freezing/thawing cycles which lead to a decrease of enzyme activities.

3.3.2. Enzyme Analysis: CAT

1. CAT (EC 1.11.1.6) activity is measured by following the decrease of absorbance of H_2O_2 at 240 nm. The enzyme assay contains 2.55 mL of 50 mM phosphate buffer (pH 7.0) and 200 μL of enzyme extract.
2. After making the blank, add 250 μL of H_2O_2 (final concentration 3.125 mM) and follow the decrease in absorption at 240 nm for 1 min. CAT activity is calculated using the molecular extinction coefficient of H_2O_2 at 240 nm ($\varepsilon = 43.6/mM/cm$).

3.3.3. Enzyme Analysis: SOD

1. Activity of SOD (EC 1.15.11) is determined by the inhibition of NBT photo-reduction. The reaction mixture contains 2.2 mL of 0.1 M potassium phosphate buffer (pH 7.8), 0.25 mL riboflavin (final concentration 1.3 μM), 0.25 mL methionine (final concentration 13 mM), 0.25 μL NBT (final concentration 63 μM), and 50 μL of enzyme extract.
2. Immerse glass test tubes containing the reaction mixture in a temperature-controlled water bath at 25°C and illuminate with a fluorescent lamp. Identical tubes, which are not illuminated, serve as blanks. Measure absorbance at 560 nm after 15 min of illumination. One unit of SOD is defined as the amount of enzyme that inhibits NBT photo-reduction to blue formazan by 50% (the formation of blue formazan is evaluated by a reaction carried out without enzyme extract).

3.3.4. Enzyme Analysis: Glutathione Reductase

1. Glutathione reductase (EC 1.6.4.2) activity is estimated by following the rate of NADPH oxidation at 340 nm. The assay mixture contains 25 μL of GSSG (final concentration 10 mM), 50 μL of $MgCl_2$ (final concentration 3 mM), 100 μL of extract, and 200 μL of 0.1 mM potassium phosphate buffer (pH 7.8).
2. Record the absorption of the blank, then start the reaction by adding 25 μL of NADPH (final concentration 0.5 mM), and follow the decrease in absorbance of NADPH at 340 nm for 15 min. Calculate GR activity using the molecular extinction coefficient of NADPH at 340 nm ($\varepsilon = 6.22/mM/cm$).

3.3.5. Enzyme Analysis: APX

1. APX (EC 1.11.1.11) activity is measured by following the decrease of ascorbate absorbance at 290 nm. The enzyme assay contains 200 μL of extract in 680 μL of 50 mM potassium phosphate buffer (pH 7.0).
2. After making the blank, add 100 μL of ascorbate (final concentration 0.5 mM) and record the absorbance at 290 nm at 25°C for 5 min. APX activity is calculated using the molecular extinction coefficient of ascorbate at 290 nm ($\varepsilon = 2.8/mM/cm$).

3.3.6. Analysis of Lipid-Soluble Antioxidants: Tocopherols and Carotenoids

1. Freeze drying (optional): For each replicate of a study-specific treatment, weigh clean seeds and place them into a small paper bag. Put the safely closed paper bag into liquid nitrogen and freeze-dry immediately. Prior to freeze drying, intermediary storage for a few weeks in a deep freezer (preferably –80°C) is possible, but care must be taken that the samples do not thaw. After freeze drying, weigh the samples and record their dry weight. Do not expose freeze-dried seeds to ambient air humidity because humidity can oxidize the analytes (i.e. transport seed samples to a balance in a desiccator over silica gel).
2. At this stage, the seeds can be ground immediately or stored. For storage, put the paper bags with the seeds into a plastic bag with silica gel, remove the air from the plastic bag by applying a vacuum (if available), and seal the plastic bag. Repeat the procedure with a further plastic bag to ensure that the samples are not destroyed if one bag leaks. The samples can now be stored in a deep freezer until grinding.
3. Sample grinding with a ball mill: Large and hard seeds may need to be roughly crushed using mortar and pestle (do not use liquid nitrogen at this stage because this wets the seeds). Small seeds can be put directly into the Teflon grinding vessel together with agate balls. To avoid warming of the sample during grinding, submerge grinding vessel containing the seeds into liquid nitrogen for a few minutes.
4. Homogenize the sample using a ball mill. For most seeds, shaking at the maximum frequency is required. The duration of grinding depends on the seed used. For pea-sized seeds, homogenization is completed within 10–15 min. Before opening, let the grinding vessel adapt to room temperature in a desiccator over silica gel to avoid condensation of air humidity on the freeze-dried powder.
5. Transfer the powder into humidity-proof vials quickly (<2 min). The material can now be stored (preferably at –80°C) until use. If treated carefully and never wetted, the seed powder can be long-term stored (we have observed no changes in antioxidant composition over 3 years in storage), but we recommend immediate analysis. Before analysis, remove the vials from the freezer and let them adapt to ambient temperature, preferably in a desiccator over silica gel, to avoid condensation of humidity on the powder after opening the vials. After withdrawing powder for one sample, close the vial immediately and return to the freezer as soon as possible.
6. Extraction (both carotenoids and tocopherols): Put ~50 mg freeze-dried seed powder (record exact weight) into a brown 1.5 mL Eppendorf micro-centrifuge tube. To neutralize acids (required for carotenoids only), add a spatula tip (approximately

50 mg) of $MgCO_3$ or $CaCO_3$. Alternatively, the organic base N-ethyldiisopropylamine is a useful solvent additive. Various solvents can be used for extraction (see Subheading 2). We recommend testing which solvent is best for your seed material. For example, add 1 mL DMSO and shake well for approximately 30 s using a vortex. Note that 100% DMSO is solid at temperatures below 18°C. Therefore, work at temperatures above 20°C. Caution: DMSO is an irritant. Work in a fume hood and wear gloves to avoid contact with skin.

7. Alternatively, freeze-dried as well as fresh seeds can be ground by hand using a mortar and pestle, followed by immediate extraction (see Note 4). Always grind under liquid nitrogen, adding a spatula tip of quartz sand to aid with grinding, if required. Never allow the sample to thaw before the addition of the extraction medium. Put ~50 mg seed material, 50 mg $MgCO_3$ or $CaCO_3$, and a small amount of liquid nitrogen into a mortar and grind rigorously to produce a fine powder. After evaporation of the liquid nitrogen, add 0.5 mL extraction medium (as above) and grind gently for ca. 30 s. Transfer the homogenate into a dark centrifuge tube, add a further 0.5 mL of extraction medium to the mortar to remove residue, and pool with the homogenate from the previous extraction.
8. Centrifuge the extract for 5 min at 4°C and 13,000 × *g*, then decant the supernatant into calibrated glass tubes, and store them in the dark. Re-suspend the pellet in 0.5 mL of solvent, vortex vigorously, and centrifuge for 20 min at 13,000 × *g* and combine supernatant with the previously obtained supernatants. Re-suspend the pellets, vortex, and centrifuge (as above) and collect supernatants until the pellet is colourless. Generally, two to three extraction steps are necessary for quantitative extraction of tocopherols and carotenoids. Further extraction steps may dilute the extract too much. Record the volume of the combined supernatants.
9. Prior to HPLC analysis, remove potential cell debris by a further centrifugation step (13,000 × *g* for 40 min) and pipette supernatants into dark auto-sampler vials.
10. For HPLC analysis of tocopherols, separate tocopherols at a constant flow rate of 1 mL/min and detect them with a fluorescence detector (excitation wavelength = 295 nm, emission wavelength = 325 nm); total run time: 16 min. The tocopherols elute in the order: δ-, γ-, α-tocopherol (Fig. 2a).
11. Prepare a series of standard solutions with varying concentrations of α-, γ-, and δ-tocopherol in DMSO (or another organic solvent), centrifuge, and analyze as described in steps 9 and 10. Calculate the amounts of tocopherols using the results of standard calibration curves, ensuring that the sample concentrations lie within the range of the calibration curve.

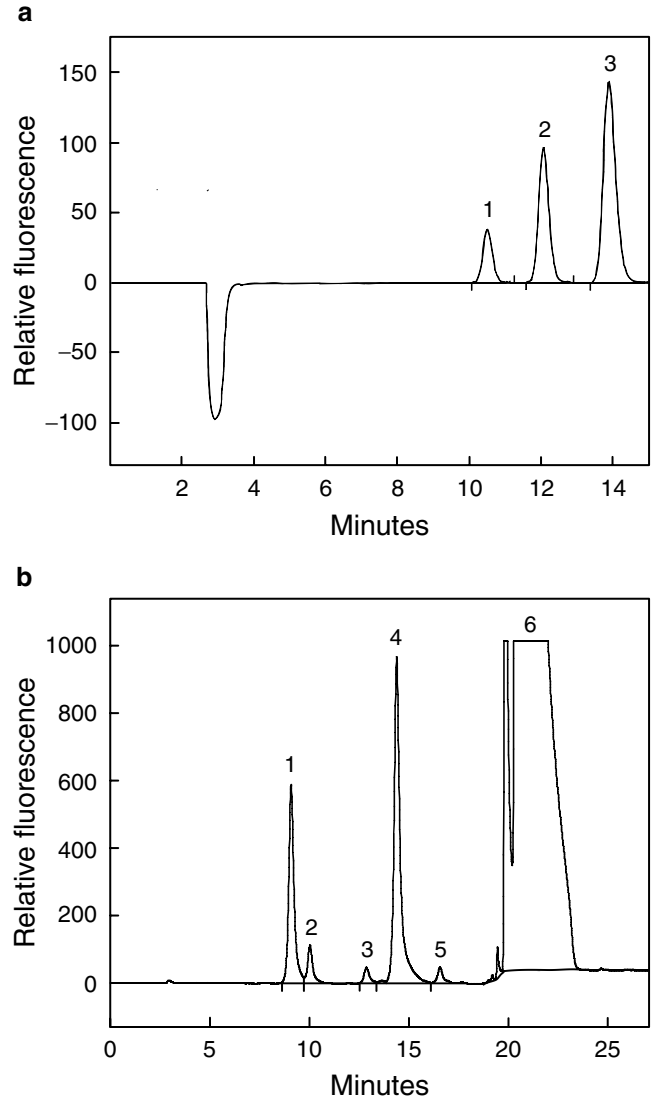

Fig. 2. (**a**) HPLC separation of tocopherols that are frequently found in seeds: 1, δ-tocopherol; 2, γ-tocopherol, and 3, α-tocopherol isolated from *Vernonia galamensis* seeds. (**b**) HPLC separation of GSH from other low-molecular-weight thiols using the monobromobimane assay: 1, cysteine; 2, γ-glutamyl-cysteine; 3, cysteinyl-glycine; 4, GSH. Peaks 1 to 4 are typically found in seed samples, here shown for *Pisum sativum* seeds. Peak 5 is the reductant DTT, and peak group 6 corresponds to monobromobimane rests that need to be removed from the column before the next injection.

12. Test the recovery of tocopherols to ensure that they are not destroyed or lost during extraction. Divide the freeze-dried seed powder into ten subsamples, and analyze five of these as described above and spike five others with known amounts of standard solution. At least 90% of the added standard should be recovered and recovery rates should be indicated in publications.

13. For HPLC analysis of carotenoids, the extract produced after step 8 can be used. Separate pigments at a constant flow rate of 1 mL/min using a gradient of 10–70% solvent B within 18 min, and then maintain 70% solvent B for 4 min (until elution of β-carotene). Equilibrate the column with 10% solvent B for 10 min before the next run. If pigments in the sample have been previously identified and a quantitative determination in seed samples is required, use a UV/vis variable detector at 440 nm. A photodiode array detector is useful for the identification of unknown pigments (record spectra of all peaks in a range from 250 to 550 nm).
14. Prepare standards of the following pigments that may be present in seeds: α- and ß-carotene, lutein, violaxanthin, antheraxanthin, zeaxanthin, and neoxanthin. If chlorophyll analysis is required, prepare standards of chlorophyll a and b. Dilute a known amount of standard in an appropriate solvent (ethanol, acetone) and analyze as described above. For the identification of unknown compounds, follow the protocols of Pfeifhofer et al. (26). Test the recovery of pigments as described in step 12.

3.3.7. Analysis of Water-Soluble Antioxidants: GSH and Glutathione Disulphide

1. Follow the advice regarding sample preparation (freeze drying, storage, and grinding) as described above under steps 1–5 of the tocopherol assay.
2. Extraction: For each replicate, extract ~50 mg (dry weight basis; record exact weight) of freeze-dried and ground or freshly ground seed material in 1 mL of 0.1 M HCl. For phenol-containing seeds (i.e. the majority of seeds), imbibe 50 mg PVPP in 1 mL of 0.1 M HCl overnight and use this mixture for the extraction of GSH from seed powder. Centrifuge for 20 min at 20,000 × *g* at 4°C. Now divide the extract and use one part of the supernatant for the determination of total GSH content (i.e. the sum of GSH + GSSG; see Note 12) and the other for the determination of GSSG (see Note 12) after blockage of GSH with NEM. If not indicated otherwise, keep extracts on ice at all times.
3. For determination of GSH, mix 120 µL of the supernatant with 180 µL of 200 mM CHES buffer and 30 µl of DTT and incubate for 60 min at room temperature to allow reduction of disulphides. Label the extract with 20 µL of 15 mM mBBr for 15 min at room temperature in the dark. It is important to note that an excess of MB must be used to ensure that all thiols present in the extract are labelled. Note also that DTT is a dithiol, so 2 mol of MB is required to label 1 mol of DTT. In addition, some oligopeptides and low-molecular-weight proteins with free cysteine residues that are not removed by centrifugation at 13,000 × *g* may also be present in the extracts and are labelled with MB. Therefore, make sure to use both DTT and MB in excess. After labelling, acidify with 250 µl of 0.25%

MSA and centrifuge for 45 min at 13,000 × *g* at 4°C. The supernatant can then be used for reversed-phase HPLC analysis (step 5). Transfer to brown auto-sampler vials.

4. For determination of GSSG, mix 400 µL of the supernatant and 600 µL of 200 mM CHES (pH 9.3) buffer with 30 µL of 50 mM NEM to block free thiols. After 15 min of incubation at room temperature, remove excess NEM by extracting five times with equal volumes of toluene. For each toluene extraction, wait until the phases have separated (if they do not separate, centrifuge very briefly), discard the toluene phase (supernatant), and then add fresh toluene. After the last toluene extraction, add 30 µL of 3 mM DTT to a 300-µL aliquot of the aqueous GSSG-containing extract (last remaining extract) and leave for 60 min at room temperature to reduce GSSG. Label with mBBr, acidify, and centrifuge as described under step 3.
5. For HPLC analysis, separate GSH from other low-molecular-weight thiols on a C18 column and detect thiols with a fluorescence detector (excitation: 380 nm, emission: 480 nm). Equilibrate the column with 12% solvent B. After injection of 20 µL of analyte, increase the concentration of solvent B to 27% in 15 min at a constant flow rate of 1 mL/min and then to 100% at 19 min in order to elute mBBr adducts. After 26 min, reset the initial solvent B concentration to 12% to re-equilibrate the column until a total run time of 35 min is reached, followed by the next injection. An example chromatogram is shown in Fig. 2b.
6. Calculate the concentrations of low-molecular-weight thiols in seed extracts using a calibration curve. Of the 1 mM stock solutions (GSH or GSH homologues) and 0.1 mM stock solutions (other thiols), add 5, 10, 15, 20, and 25 µL to 395, 390, 385, 380, and 375 µL 0.1 M HCl, respectively, so that the total volume is 400 µL. Also prepare blank samples that contain only 400 µL of 0.1 M HCl. Mix the diluted standards or the blank sample with 600 µL of 200 mM CHES (pH 9.3) buffer. Of this mixture, treat 300 µL with 30 µL of 3 mM DTT, leave for 60 min at room temperature, then label with mBBr, acidify, and centrifuge as described under step 3.
7. Test the recovery of GSH as described for other HPLC analyses in this chapter.
8. In addition to evaluating seed GSH and GSSG contents (usually in the range of nmol/g DW), we recommend that you also calculate the GSH half-cell reduction potential ($E_{GSSG/2GSH}$), which is a superior stress marker compared to ratios of GSSG/GSH or GSSG/(GSH + GSSG) (24, 29). Calculations of $E_{GSSG/2GSH}$ follow the Nernst equation:

$$E_{\mathrm{GSSG/2GSH}} = E^{0'} - \frac{RT}{nF}\ln\frac{[\mathrm{GSH}]^2}{[\mathrm{GSSG}]}$$

where R is the gas constant (8.314 J/K/mol); T, temperature in K; n, number of transferred electrons ($2\mathrm{GSH} \rightarrow \mathrm{GSSG} + 2\mathrm{H}^+ + 2\mathrm{e}^-$); F, Faraday constant (9.6485×10^4 C/mol); and $E^{0'}$, standard half-cell reduction potential of GSH at pH 7 ($E_{\mathrm{GSSG/2GSH}}{}^0 = -240$ mV). [GSH] and [GSSG] are molar concentrations of GSH and GSSG, which can be estimated using seed water contents that must be determined prior to freeze drying. The density of water can be approximated as ca. 1 g/mL, and the amount of water per gram seed is used in the calculations of [GSH] and [GSSG].

3.3.8. Analysis of Water-Soluble Antioxidants: Ascorbate and Dehydroascorbate

1. Follow the advice regarding sample preparation (freeze drying, storage, and grinding) as described above under steps 1–5 of the tocopherol assay.
2. For extraction, homogenize ~50 mg (dry weight basis; record exact weight) of freeze-dried and ground or freshly ground seed material in 1 mL 1.5% (w/v) metaphosphoric acid containing 1 mM EDTA. If not indicated otherwise, keep extracts on ice and proceed with the derivatization as soon as possible.
3. Adjust an aliquot (700 μL) of the extract to neutral pH by the addition of 450 μL of 0.2 M aqueous Tris in a brown Eppendorf micro-centrifuge tube, and then add 10 μL of freshly prepared 1,2-phenylenediamine dichloride solution to derivatize DHAA. Derivatization is required to produce a DHAA adduct with an absorption maximum at a higher wavelength. Underivatized DHAA absorbs at low UV wavelengths at which most organic solvents also strongly absorb, preventing the analysis of underivatized DHAA. Vortex for 15 s, incubate for 25 min at room temperature in the dark, then stop the reaction by the addition of 85% ortho-phosphoric acid (10 μL), and keep the extract on ice until centrifugation at $13{,}000 \times g$ and 4°C for 20 min. Transfer the extract to a brown auto-sampler vial.
4. For HPLC analysis, carefully equilibrate the column (this is best conducted overnight at a low flow rate, such as 0.1 mL/min). Before the first injection, increase the flow rate gradually to 1 ml/min. At a flow rate of 1 ml/min, inject 20 μL of the analyte. Using a photodiode array detector, detect peaks simultaneously at two wavelengths (348 and at 248 nm). After about 7 min, AA can be detected at 248 nm and DHAA can be detected as a DHAA-1,2-phenylenediamine derivative at 348 nm, eluting after the peak of underivatized 1,2-phenylenediamine dichloride (Fig. 3). Set the total analysis time to 20 min to elute co-extracted seed compounds before the next injection. Note that the extracts degrade rapidly and need to be injected

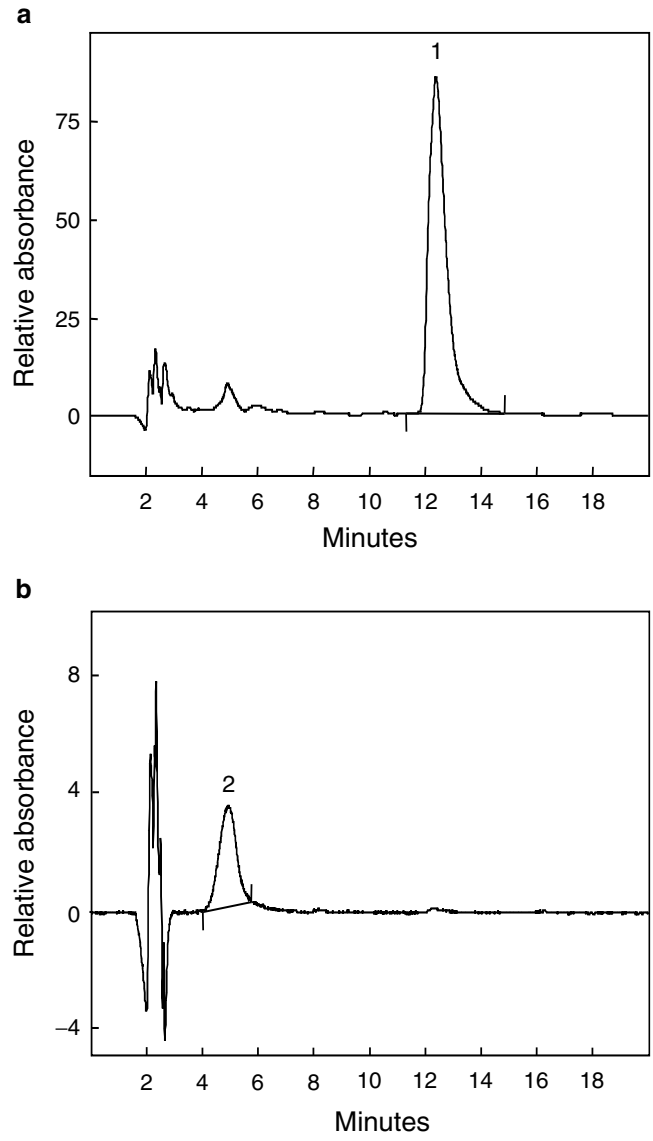

Fig. 3. HPLC separation and simultaneous detection of AA and DHAA isolated from *Quercus robur* seeds. (**a**) Ascorbate (peak 1) is detected at 248 nm. (**b**) The 1,2-phenylenediamine derivative of dehydroascorbate (peak 2) is detected at 348 nm.

as soon as possible and kept on ice until injection (or in a cooled auto-sampler below 4°C).

5. Prepare a series of standard solutions with varying concentrations of AA and DHAA (see Note 13), derivatize, and analyze as described in steps 3 and 4. Calculate the concentrations of AA and DHAA using the results of standard calibration curves, ensuring that the sample concentrations lie within the range of the calibration curve.

6. Test recovery rates as described for other HPLC analyses in this chapter. In addition, check the loss of AA due to the derivatization procedure by comparison of underivatized with derivatized extracts.
7. If required, calculate the half-cell reduction potential of the AA/DHAA couple as described for the GSH/GSSG redox couple, but note that the number of transferred electrons is 1 rather than 2. Therefore, expressing the redox state as a ratio may be sufficient for most studies.

3.4. Markers of Oxidative Stress

3.4.1. Markers of Lipid Peroxidation: MDA

1. Homogenize seeds (~0.5 g DW) in a chilled mortar with 5 mL of TBA–TCA solution. Boil the homogenate for 30 min at 95°C in a water bath and then quickly cool on ice.
2. Centrifuge the homogenate at 16,000 × *g* for 30 min and use 2 mL of the obtained supernatant for MDA determination. MDA equivalents are calculated from the difference in absorbance at 532 and 600 nm using an extinction coefficient of 155/mM/cm.

3.4.2. Markers of Lipid Peroxidation: Conjugated Dienes

1. Grind ~10 seeds (for sunflower-sized seeds) in a mortar in the presence of liquid nitrogen, and mix 100 mg of the obtained powder with 3 mL of chloroform and 3 mL of 5 mM EDTA stabilized with 1% (w/v) NaCl. Vortex the mixture and centrifuge at 2,000 × *g* for 15 min.
2. Collect the bottom phase and dry at 45°C under a stream of gaseous nitrogen. Add 1 mL of chloroform, then dry a 10-µL aliquot of this mixture under a stream of nitrogen, and resuspend in 1 ml of ethanol. Record the absorption spectrum from 200 to 300 nm. Conjugated dienes concentration is calculated using the absorption at 233 nm (ε = 27/mM/cm).

4. Notes

1. We recommend using a 500 W lamp at a minimum. Lower irradiances slow down the reaction rate. Attention must also be paid to ensure that each test tube receives the same amount of light.
2. Frequently, 80% acetone is used as a solvent for pigment analysis. However, the water added to the acetone can dissolve acids that can destroy chlorophylls and some carotenoids. We strongly recommend the use of DMSO, pure acetone, ethanol, or other solvents described. In addition, work in dim light and use dark centrifuge tubes and auto-sampler vials to avoid pigment destruction by light during extraction and handling. Because of the thermolability of many carotenoids, do not heat samples.

3. For some seed materials, oxidation of the analytes may be a problem. To avoid chemical reactions during sample storage or grinding, we strongly recommend freeze drying of seeds prior to analyte extraction. Alternatively, samples can be ground in liquid nitrogen using a mortar and pestle, but meticulous care must be taken to avoid thawing during sample preparation. For example, chlorophyll can be degraded to phaeophytin by co-extracted acids either when using organic solvents containing water for extraction (see above) or when wet seeds are analyzed. The addition of an antioxidant to the extraction solvent can be helpful, for example butylated hydroxytoluene. Similarly, GSH and AA are rather reactive and unstable in the presence of oxidizing conditions.
4. We recommend the use of a ball mill for homogenization of samples for the following reasons. Firstly, no material is lost during extraction (as may occur during grinding with a mortar and pestle). Secondly, provided that samples are freeze-dried before grinding, the ground samples can be stored for long periods. Thirdly, if large numbers of replicates need to be analyzed, grinding by hand is very tedious. Especially for investigations of more than one antioxidant, the availability of ground powder that can be stored between analyses is very helpful. Using the same powder for different assays also allows better statistical correlation of the data. Moreover, we recommend using grinding vessels made of chemically inert materials, such as Teflon, and agate balls rather than stainless steel vessels and grinding balls because antioxidants can react with metals, potentially interfering with the analyses.
5. The HPLC system described is useful for the protocol given, but in more advanced labs, other systems can also be used, e.g. HPLC-MS potentially with different columns that have smaller particle sizes and lengths or UPLC-MS, requiring amendment of the method.
6. Caution: All thiol-blocking agents are toxic. Handle with care and wear gloves.
7. We recommend a system with two pumps with solvent mixing under high pressure rather than low-pressure-mixing single pumps because the mixing volume is smaller in the first case. Therefore, more precise gradients can be achieved which is advantageous for this method, although good low-pressure-mixing single pumps may also be able to achieve a sufficiently accurate gradient.
8. The term seeds used here (and throughout the methods) is generic. We highly recommend to remove the external structures of the seed (such as the pericarp or testa) because they are often rich in phenolic compounds, which can scavenge ROS

during extraction, react with antioxidants, or bind to proteins and alter enzyme activities. We also recommend working with "fresh" seeds because freezing/thawing may lead to ROS formation. Attention must also be paid to the mode of expression of the results: it is best to express the amount of ROS per gram DW, in particular if studying the ROS content during seed development or germination, i.e. when seed fresh weight and protein concentrations change.

9. The neutralization step is the most critical one in this protocol. It has to be done very carefully so that the pH of 7.5–7.6 is not exceeded, although a slightly too high pH value can be overcome by adding perchloric acid. It is important that all samples are equilibrated with the same volume of KOH.
10. It may be necessary to centrifuge the incubation solution (e.g. at 16,000 × *g* for 2 min) or filter it through a 0.2-μm pore filter before measurements to remove suspended interfering high-molecular-weight compounds. Both the epinephrine and the XTT assays are convenient and rapid methods for the quantification of extracellular $O_2^{\bullet -}$ production. However, it cannot be excluded with certainty that both reagents react with other ROS or RNS in seed leachates. Therefore, the specificity of the assays must be tested. If the $O_2^{\bullet -}$ production is considerably decreased in the presence of SOD, this is a good indication for assay specificity. In studies for which the precise identity of the ROS is important, confirmation must be sought by more sophisticated methods, such as electron paramagnetic resonance (EPR), that lie outside the scope of this book.
11. Contrary to ROS measurements, seeds can be frozen (if possible at –80°C) or freeze-dried before preparing protein extracts without altering enzyme activities.
12. The assay described for "GSH" also detects cysteine, γ-glutamylcysteine, cysteinyl-glycine, and GSH homologues and that for "GSSG" the corresponding disulphides. For simplification, we use the terms "GSH" and "GSSG".
13. DHAA is very unstable in the presence of oxygen. After the removal of an aliquot required for the preparation of a standard stock solution, we recommend flushing the vial with the remaining chemical with gaseous nitrogen before further storage.

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Chapter 21

Protein Damage and Repair Controlling Seed Vigor and Longevity

Laurent Ogé, Caroline Broyart, Boris Collet, Béatrice Godin, Denis Jallet, Gildas Bourdais, Dominique Job, and Philippe Grappin

Abstract

The formation of abnormal isoaspartyl residues derived from aspartyl or asparaginyl residues is a major source of spontaneous protein misfolding in cells. The repair enzyme protein L-isoaspartyl methyltransferase (PIMT) counteracts such damage by catalyzing the conversion of abnormal isoaspartyl residues to their normal aspartyl forms. Thus, this enzyme contributes to the survival of many organisms, including plants. Analysis of the accumulation of isoaspartyl-containing proteins and its modulation by the PIMT repair pathway, using germination tests, immunodetection, enzymatic assays, and HPLC analysis, gives new insights in understanding controlling mechanisms of seed longevity and vigor.

Key words: Isoaspartyl methyltransferase, Arabidopsis, Seed, Longevity, Vigor

1. Introduction

Under physiological conditions, cellular proteins are subjected to nonenzymatic isomerization and/or deamidation of aspartyl and asparaginyl residues, respectively. These spontaneous reactions give rise to the formation of an abnormal isoaspartyl residue via a succinimide intermediate (cyclic imide) (1). This damage can affect the physicochemical properties and biological activity of proteins (2, 3) since the peptide backbone is transferred from the alpha-carboxyl group to the side-chain beta-carboxyl (Fig. 1). Protein L-isoaspartyl methyltransferase (PIMT; EC 2.1.1.77) is a highly conserved enzyme that can specifically recognize proteins containing altered aspartyl residues. PIMT catalyzes the transfer of a methyl group from *S*-adenosyl methionine (AdoMet) to the free

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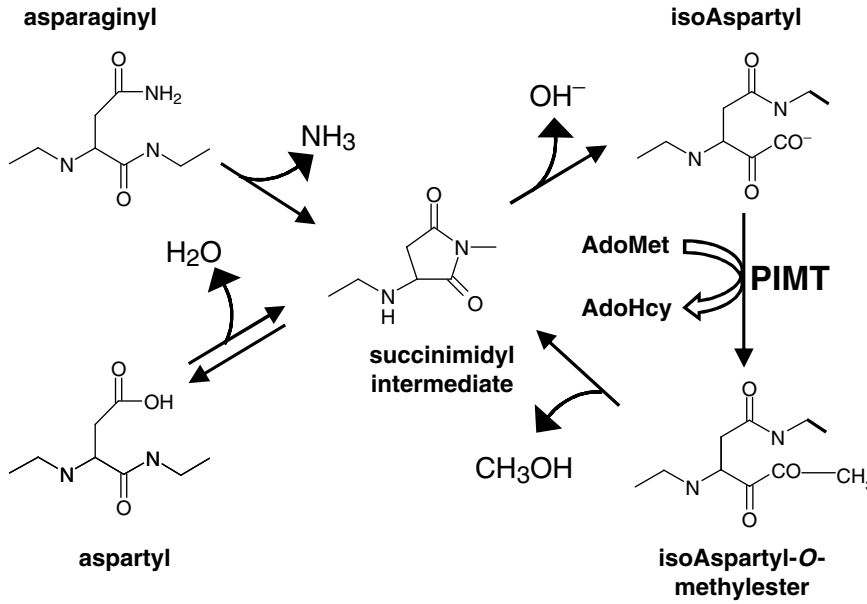

Fig. 1. Mechanism of spontaneous isoaspartyl formation and enzymatic repair by PIMT. Deamidation of asparaginyl (*upper left*) and isomerization of aspartyl (*bottom left*) lead to the formation of an unstable succinimidyl ring that is spontaneously hydrolyzed to generate a mixture of aspartyl and of abnormal isoaspartyl residues at physiological pH. PIMT catalyzes the first step of the conversion of isoaspartyl residues into aspartyl residues. AdoMet, *S*-adenosyl methionine; AdoHcy, *S*-adenosyl homocysteine.

carboxyl groups of abnormal aspartyl residues in proteins, leading to the repair of damaged proteins and peptides (Fig. 1) (4, 5). The protective role of PIMT during aging or upon deleterious conditions has been well-characterized using genetic approaches in *Escherichia coli* (6, 7), mice (8, 9), *Drosophila* (10) and nematodes (11). In plants, most of the PIMT activity has been found in the seed (12, 13). Intriguingly two PIMT genes, *PIMT1* and *PIMT2*, that are both expressed in the seed have been identified in Arabidopsis (14, 15). Recent genetic analysis (16) has shown that at least the PIMT1 repair pathway contributes importantly to seed longevity and germination or seedling vigor in limiting the accumulation of isoaspartyl residues in proteins. This study illustrated how quantification of isoaspartyl-containing peptides, detection of PIMT proteins and activity using High-Performance Liquid Chromatography (HPLC), western blotting, and enzymatic assay tools are helpful to investigate regulatory mechanisms involved in seed responses to stressful environments. These stress conditions that adversely affect the germination rate of low vigor seed lots can be mimicked in the laboratory by a controlled deterioration treatment (CDT) or by high salt or osmotic stress such as mannitol treatment. All these tools can allow a better understanding of protective mechanisms involved in seed quality. Furthermore, these tools are useful as biochemical markers to evaluate the quality of the seed lots through storage and long-term conservation.

2. Materials

2.1. Seed Production

1. Plant substrate (Tref Peat Substrates with N/P/K = 17/10/14, pH 6.2, Tref, The Netherlands).
2. 24-cell trays (75-mm diameter, 70-mm deep per cell) (Puteaux, France).
3. Phytosanitary solution (PS solution): 5 mL/L fungicide Previcur N (Bayer CropScience, France) and 400 mg/L insecticide Hortigard (Syngenta Agro, France) solubilized in water.
4. A regulated growth chamber: photoperiod, 16-h fluorescent light period (250 μmol/m²/s and 8 h of darkness); temperature (light/dark: 21°C/19°C); relative humidity of 50–60%.

2.2. Preparation of Seed Samples

1. Sterilization solution 1 (SS1): one tablet of bleach (1.5 g chlorine per tablet) (Bernard, France) dissolved in 40 mL of distilled water.
2. Sterilization solution 2 (SS2): 5 mL of SS1 in 45 mL of 96% ethanol.
3. 100% ethanol.
4. Coprology boxes, 150 mL (CML, France).
5. Laminar flow hood.

2.3. Controlled Deterioration Treatment

1. Transparent airtight tube carriers (Fisher Scientific).
2. $MgCl_2$ saturated solution: 100 g of $MgCl_2$ solubilized in 200 mL of water. Continue adding $MgCl_2$ until the solution reaches saturation.
3. Oven regulated at 40°C.
4. Temperature and humidity digital controller (e.g., Kimo, France).

2.4. Seed Germination

2.4.1. Sterilization of Solutions

1. Syringes (10 mL) without needle.
2. Puradisc 30 syringe filters (0.2 μm pore size) (Whatman).
3. 5 M NaCl: 2.9 g of NaCl is dissolved in 10 mL of distilled water. This solution is stored at RT.
4. 5 M mannitol: 9.1 g of mannitol is dissolved in 10 mL of distilled water. This solution is stored at RT.
5. Laminar flow hood.

2.4.2. Seed Germination on Agar Medium Supplemented with NaCl or Mannitol

1. Seed germination medium (GM): 7 g/L agar HP 696 (Kalys, France) and 580 mg/L MES, pH 5.7, solubilized in water and autoclaved.
2. Sterilized 5 M NaCl and 5 M mannitol solutions.

3. GM supplemented with 100 mM NaCl, 200 mM NaCl, 300 mM mannitol, or 400 mM mannitol. Dilute filter-sterilized stocks noted in item 2 (above) in the GM maintained at 60°C prior to agar solidification.
4. Petri dishes (55-mm diameter).
5. Parafilm.
6. Binocular microscope (Leica, France).
7. A phytotronic chamber (e.g., Sanyo, France) with a 16-h photoperiod at 25°C (light period)/20°C (dark period) and a constant 70% RH (standard conditions).

2.5. Protein Extraction

Protein extraction can be performed using different buffers according to the analyses that follow. The three ways described below use some common products that we detail here.

1. Porcelain mortar (75-mm diameter) and pestle (23-mm diameter, 84-mm length).
2. Ice and liquid nitrogen.
3. Protein Assay Dye Reagent Concentrate (Bio-Rad).
4. Bovine serum albumin is dissolved in an appropriate volume of water to obtain 1 μg/μL concentration. This solution is stored in 60-μL aliquots at –20°C.

2.5.1. Preparation of Plant Homogenates for Protein Blot Analyses

1. Complete Mini Protease Inhibitor Cocktail (Roche): 1 tablet is solubilized in 1.5 mL of distilled water and stored in 100-μL aliquots at –20°C.
2. Extraction buffer 1: 100 mM HEPES, pH 7.5, 500 μM EDTA 100 μM dithiothreitol (DTT) (Invitrogen), 10% (w/v) sucrose, and 1% (v/v) Complete Mini Protease Inhibitor Cocktail. This solution is stored at RT.

2.5.2. Preparation of Plant Extracts for Measuring Seed PIMT Methyltransferase Activity

1. Extraction buffer 2: 100 mM pH 7.5, 10 mM 2-mercaptoethanol, 10 mM sodium hydrosulfite, 10 mM sodium metabisulfite, 10% (v/v) glycerol, 1 μM leupeptin, and 1 mM phenylmethylsulfonyl fluoride (PMSF). This solution is stored at RT.

2.5.3. Preparation of Plant Homogenates for L-Isoaspartyl Quantitation

1. Extraction buffer 3: 100 mM Tris–HCl, pH 8.0.
2. Pieces of Miracloth paper (2×2 cm) (Calbiochem).
3. Syringes (1 mL) without needle.
4. Ammonium sulfate.

2.6. Protein Gel Blot Analyses

1. Mini-PROTEAN® 3 Cell (Bio-Rad) and PowerPac™ HC power supply (Bio-Rad).
2. Modified Laemmli (17) buffer (2×): 0.125 M Tris–HCl, 20% (v/v) glycerol, 4% (w/v) sodium dodecyl sulfate (SDS), 10% (v/v) 2-mercaptoethanol, and 0.03% (w/v) bromophenol

2.6.1. One-Dimensional SDS-Polyacrylamide Gel Electrophoresis

blue, pH 6.8. This solution is stored at –20°C in 9-mL aliquots without 2-mercaptoethanol that is added when extraction is performed.

3. Separating buffer (4×): 1.5 M Tris–HCl, pH 8.8, and 0.4% (w/v) SDS. This solution is stored at RT.
4. Stacking buffer (4×): 0.5 M Tris–HCl, pH 6.8, and 0.4% (w/v) SDS. This solution is stored at RT.
5. 30% (w/v) acrylamide/bis solution (Bio-Rad) and *N,N,N,N*′-Tetramethyl-ethylenediamine (TEMED) (BioRad).
6. 10% (w/v) ammonium persulfate (APS) solution: 1 g of APS (GE Healthcare) is dissolved in 10 mL of distilled water and stored as single-use aliquots (150 µL) at –20°C.
7. Running buffer (5×): 125 mM Tris, 960 mM glycine, and 0.5% (w/v) SDS. pH is then adjusted to 8.3. This solution is stored at RT.
8. Isopropyl alcohol.
9. Prestained protein weight marker (Euromedex).

2.6.2. Coomassie Staining

1. Coomassie Brilliant Blue R250 Staining Solution (Bio-Rad).
2. Coomassie Brilliant Blue R250 Destaining Solution (Bio-Rad).
3. Rocking platform STUART® (Keison Products).

2.6.3. Western Blot Analyses

Specific amino acids sequences from PIMT1 (18 amino acids: C-LQVVDKNSDGSVIKDET) and PIMT2 (11 amino acids: SGT GSSGKRG-C) proteins were synthesized and used to immunize rabbits. The antisera were immunoaffinity-purified against their corresponding peptides bound to Sepharose matrix.

1. Specific anti-PIMT1 and anti-PIMT2 antibodies.
2. Anti-Rabbit IgG (whole molecule)–Peroxidase antibody produced in goat (Sigma-Aldrich).
3. Trans-Blot SD semidry transfer cell (Bio-Rad).
4. Nitrocellulose membrane Hybond-C Extra sheets cut in 6.5 × 8.5-cm pieces (GE Healthcare).
5. 3MM Chr sheets cut in 6.5 × 8.5-cm pieces (Whatman).
6. PBS-T buffer (10×): 1.4 M NaCl, 27 mM KCl, 81 mM Na_2HPO_4, 150 mM KH_2PO_4, and 0.03% (v/v) Tween 20, pH 7.6. This solution is stored at RT.
7. Enhanced chemiluminescent (ECL) reagents ECL™ Advance Western Blotting Detection Kit (GE Healthcare).
8. Blocking buffer: 2% (w/v) ECL™ Advance Blocking Agent (GE Healthcare) in 1× PBS-T buffer.
9. Radiographic hyperfilm ECL (GE Healthcare).

10. Saran wrap.
11. X-ray film cassette (18×24 cm).
12. X-ray developer solution (Kodak LX24) and X-ray fixer solution (Kodak AL4).
13. Rocking platform STUART® (Keison Products).
14. Dark room.

2.7. Measurement of PIMT Methyltransferase Activity

1. 100 μM *S*-adenosyl (methyl-^{14}C)methionine (52 mCi/mmol; Perkin-Elmer).
2. 0.2 M sodium citrate solution: 588 mg sodium citrate is dissolved in 10 mL distilled water and the pH is adjusted to 6.0. This solution is stored at RT.
3. 2 mM albumin from chicken egg white (ovalbumin) Grade V (Sigma-Aldrich) solution: 900 μg protein is dissolved in 10 mL of 0.2 M sodium citrate solution. This solution is stored as 100-μL aliquots at –20°C.
4. Stop solution: 0.2 M sodium hydroxide and 1% (w/v) SDS.
5. 3MM Chr sheets (1×8.5 cm).
6. 20-mL counting vials.
7. ACS-II scintillation cocktail (GE Healthcare).
8. Safety equipment: chemical hood, benchcoat.
9. Scintillation counter 1409 (Perkin Elmer previously Wallac).
10. Oven regulated at 37°C.

2.8. Quantification of Damaged Aspartyl and Asparaginyl Residues

2.8.1. Repair Reaction

1. ISOQUANT® Isoaspartate Detection Kit (Promega) including: 5× Reaction buffer (0.5 M sodium phosphate, pH 6.8, 5 mM EGTA, 0.02% (w/v) sodium azide, and 0.8% (v/v) Triton X-100), AdoMet, *S*-adenosyl homocysteine (AdoHcy), isoaspartyl-containing peptide, PIMT enzyme, stop solution (0.3 M phosphoric acid).
2. 300-μL plastic vials (Chromacol, UK) and caps (Interchim, France).
3. Water bath regulated at 30°C.

2.8.2. HPLC and UV Detection of AdoHcy

1. HPLC: Beckman Coulter System Gold 508 Autosampler, 126 Solvent Module, 168 Diode Array Detector (DAD).
2. All-glass filter holder (47-mm diameter) (Millipore).
3. Nylon membrane filters (47-mm diameter; 0.45 μm pore size) (Whatman).
4. Potassium phosphate buffer stocks: 1 M KH_2PO_4 (136 g dissolved in 1 L of distilled water) and 1 M K_2HPO_4 (174 g dissolved in 1 L of distilled water). These stock solutions are

autoclaved and stored at RT. For 1 L of 50 mM potassium phosphate buffer, pH 6.2, 40.4 mL of 1 M KH_2PO_4 stock solution, 9.6 mL of 1 M K_2HPO_4 stock solution, and 950 mL of distilled water are mixed together. This solution is prepared extemporaneously.

5. 100% methanol HPLC-gradient.
6. Synergi 4 μm Hydro-RP 80Å 150×4.6 mm column (Phenomenex) associated with a SecurityGuard guard cartridge (Phenomenex). Filters for cartridge (Phenomenex).
7. Vacuum pump.

3. Methods

3.1. Seed Production

1. Nondormant seeds from Wassilewskija (Ws) ecotype are sown in a 24-cell tray containing substrate pretreated with 1 L of PS solution for seed production (2 seeds per cell).
2. Plants are watered three times a week throughout a 60-day period to obtain full maturation of seeds on mother plants.
3. Watering is stopped for 3 weeks to allow complete seed maturation and desiccation before harvest. Seeds are stored in 15-mL tubes at 4°C before protein sample preparation.

3.2. Preparation of Seed Samples

1. Mature seeds are harvested after full maturation from dehydrated siliques.
2. The collected seeds are systematically sterilized under a laminar flow hood. Aliquots of seed samples are placed into 1.5-mL tubes (approximately 10 mg per tube) containing 1 mL of SS2 and stored for 10 min at RT with occasional shaking. The solution is discarded and the seeds are washed twice with 1 mL of 100% ethanol. Then, the solution is discarded and the tubes are stored open at room temperature for 8 h until complete drying of seeds occurs.
3. The sterilized seeds are either used immediately in the dried state or are stored in plastic coprology boxes at 7°C, 40% RH, in a storage cabinet.

3.3. Seed Germination

3.3.1. Sterilization of Solutions

1. 5 M NaCl and 5 M mannitol solutions are prepared by solubilizing appropriate amounts of powder with distilled water.
2. Solutions are filter-sterilized using a syringe under a laminar flow hood.
3. The sterilized solutions are collected in sterile bottles and serve as the stock solutions.

3.3.2. Chemical Treatments to Test Seed Vigor

1. Sterilized seeds are sown in Petri dishes containing 7 mL of the appropriate GM supplemented with 100 mM NaCl, 200 mM NaCl, 300 mM mannitol, or 400 mM mannitol.
2. Petri dishes are sealed with parafilm and stratified for 2 days at 4°C and then incubated in a phytotronic chamber maintained at germination conditions.
3. Germination measurements are scored one to two times per day using a binocular microscope. Seeds are considered germinated as soon as endosperm rupture is observed.

3.4. Controlled Deterioration Treatment Tests

3.4.1. Controlled Deterioration Treatment

The CDT conducted at high RH and described in Chapter 14 is a powerful tool to investigate the quality of a seedlot (16, 18–20). We describe here a milder protocol conducted at low RH that allows a decrease in seedlot viability over weeks instead of days. The RH is achieved using an appropriate saturated salt solution and temperature (21). The protocol may be adjusted according to the seed species or the severity of aging. In the case of high RH conditions, seeds must be dried back as described in Tesnier et al. (20) before sowing.

1. Hundred milligrams of aliquots of surface sterilized seeds are placed into a rack, and the storage treatment is performed for various times (from 2 to 12 weeks) by storing the seeds at 40°C in the oven and 32% RH in an airtight tube carrier containing a saturated solution of $MgCl_2$. Temperature and relative humidity are controlled inside the transparent airtight tube carrier using a digital controller (see Note 1).
2. After each period of storage, tubes are removed from the airtight tube carrier under a laminar flow hood and stored at 4°C before running germination experiments.

3.4.2. Germination Kinetics

An example of the germination capacity of Arabidopsis seeds ecotype Wassilewskija subjected to CDT is presented in Fig. 2. Twelve weeks are necessary for deterioration of this seedlot, but this result depends on several parameters, such as the culture conditions of the mother plants.

1. Artificially aged seeds are sown in Petri dishes containing 7 mL of GM. Petri dishes are sealed with parafilm and then incubated in a phytotronic chamber maintained at germination conditions.
2. Germination is measured as described above (see Subheading 3.3.2).

3.5. Protein Extraction

3.5.1. Preparation of Plant Homogenates for Protein Blot Analyses to Detect PIMT

1. Freshly harvested dry mature seeds (100 mg) and 1-day imbibed seeds (100 mg) are ground in liquid nitrogen using a mortar and pestle. The powder is transferred in a new mortar containing 300 μL of extraction buffer 1 and ground further.

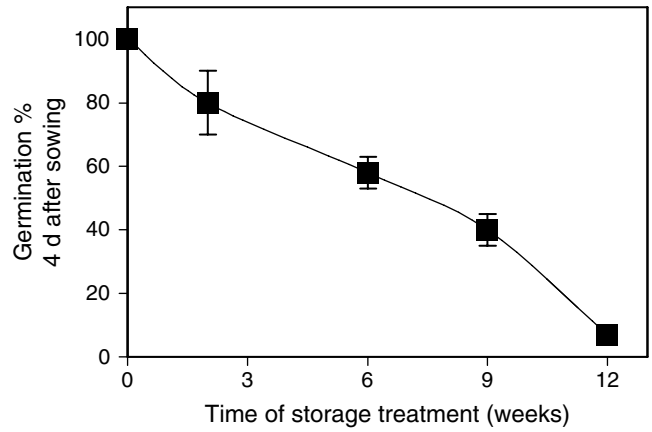

Fig. 2. Sensitivity of Ws seeds to storage treatment conducted at 40°C and 32% RH. Germination percentages were measured 4 days after sowing. Values are from four replicates of 100 seeds each (4 × 100) (mean ± SD).

2. The slurry is centrifuged at 16,000 × *g* at 4°C for 10 min and the resulting supernatant is stored at −20°C.
3. Protein concentration is determined using the Bradford protein assay (22). Bovine serum albumin is used as a standard.

3.5.2. Preparation of Plant Homogenates for PIMT Activity Assay

1. Dry mature seeds (100 mg) are ground in liquid nitrogen with a chilled mortar and pestle.
2. The seed powder is homogenized in 1 mL of extraction buffer 2 and the suspensions are centrifuged at 14,000 × *g* for 10 min at 4°C, and the supernatants are collected.
3. Protein concentration is determined using the Bradford protein assay. Bovine serum albumin is used as a standard.
4. These soluble extracts are used as the source of the PIMT enzyme and stored in 60-µL aliquots at −80°C.

3.5.3. Preparation of Plant Homogenates for L-Isoaspartyl Quantification

1. Dry mature seeds (100 mg) and one day imbibed seeds (100 mg) are ground in liquid nitrogen with a chilled mortar and pestle.
2. The powder is homogenized with 1.6 mL of extraction buffer 3.
3. The slurry is centrifuged at 16,000 × *g* at RT for 10 min and the resulting supernatant is filtered by gravity through Miracloth paper inserted into a 1-mL syringe (compressed at the bottom).
4. Collected proteins are precipitated by 65% (w/v) ammonium sulfate (see Note 2). Typically, an amount of 410 mg of ammonium sulfate is dissolved in 1 mL of the protein extract. Following incubation for 1 h at 4°C, precipitated proteins are collected by centrifugation at 16,000 × *g* at 4°C for 15 min.

5. The pellets are resuspended in 150 µL of 100 mM Tris–HCl, pH 8 and stored at –20°C.
6. Protein concentration is determined using the Bradford protein assay. Bovine serum albumin is used as a standard.

3.6. Protein Gel Blot Analyses

3.6.1. One-Dimensional SDS-Polyacrylamide Gel Electrophoresis

1. Gels are hand cast with a short plate and a spacer plate (0.75-mm thick). Ten percent separating gel solution is prepared (for two 8×6 cm gels) by mixing 500 µL of the 4× separating buffer, with 2.8 mL acrylamide/bis solution, 3.6 mL water, 70 µL APS solution, and 2.8 µL TEMED. Separating gel solution is poured, leaving space for the stacking gel solution (approximately 5 mm below the well) and overlaid with 1 mL of isopropyl alcohol. The running gel is polymerized in about 30 min (see Note 3).
2. Stacking gel solution is prepared (for two 8×6 cm gels) by mixing 100 µL of the 4× stacking buffer, with 420 µL acrylamide/bis solution, 2 mL water, 25 µL APS solution, and 2.5 µL TEMED. Isopropyl alcohol is removed from the plates, the stacking gel solution is loaded and the comb is inserted. The stacking gel is polymerized in about 30 min (see Note 3).
3. Once the stacking gel is polymerized, the comb is carefully removed (see Note 4). The components of the Mini-PROTEAN 3 Electrophoresis Module Assembly are all assembled, and the gels are placed into the tank.
4. Running buffer is prepared by diluting 100 mL of 5× running buffer with 400 mL of water. This buffer is added to the upper and lower chambers of the gel unit (see Note 5).
5. A 20-µL mixture composed of 10 µL of protein (10 µg) and 10 µL of 2× Laemmli buffer is loaded in each well. 5 µL of prestained molecular weight markers are loaded in a well to estimate protein molecular weights.
6. The lid is placed onto the module and connected to the PowerPac™ HC power supply. Gels run for 30 min at 10 mA/gel through the stacking gel and 1.5 h at 25 mA/gel through the running gel.

3.6.2. Coomassie Blue Staining

1. The gel is transferred in a plastic box containing 15 mL of Coomassie solution and rocked for 1 h at RT.
2. The Coomassie solution is discarded, and the gel is rinsed five times with distilled water.
3. The gel is washed with destain solution until proteins appear.
4. The gel is kept in distilled water at 4°C until use.

3.6.3. Western Blotting for Detection of PIMT Proteins

1. One-dimensional separated proteins (10 µg of protein per lane) are transferred to nitrocellulose membrane. For this, the stacking gel is removed and a gel "sandwich" is constructed,

composed of the nitrocellulose membrane and separating gel surrounded by four sheets of Whatman paper (two sheets above and two sheets below) paying attention to eliminate all air bubbles inside. Make sure that all components are presoaked with 1× running buffer.

2. Proteins are transferred using a semidry transfer cell at 170 mA for 45 min.
3. The nitrocellulose membrane is removed (see Note 6), air-dried for 30 min to increase protein binding, and the nonspecific binding sites are blocked overnight under gentle rocking at 4°C in a box containing 30 mL of blocking buffer.
4. The blocking buffer is discarded, and the blot is incubated for 5 h at 25°C under gentle rocking with the primary antibody in 20 mL of blocking buffer (The quantity of antibody depends on the specificity; we use a 1:10,000-fold dilution).
5. The membrane is washed three times for 10 min each with 20 mL of PBS-T and incubated for 2 h under rocking at 25°C with 1.5 μL of goat anti-rabbit peroxidase-conjugated antibodies (1:13,000-fold dilution) in 20 mL of PBS-T.
6. The secondary antibody is discarded, and the membrane is washed twice for 15 min each with PBS-T to remove any unbound conjugate. Then, the membrane is stored in distilled water.
7. Chemiluminescence of the signal is developed using an ECL kit. For this aim, the blot is covered by substrate preparation (0.1 mL/cm^2) and incubated for 1 min at RT (see Note 7). The blot is removed, excess detection reagent is drained off and the blot is placed in plastic wrap to prevent contact with radiographic film. All air bubbles should be removed.
8. In a dark room using a red safelight, the blot is exposed with the protein side up for 1 min (or less/more time, depending on the signal intensity) to autoradiography film in an X-ray film cassette.
9. The film is developed using developer and fixer solutions. An example of the results obtained with protein extracts from dry and 1-day imbibed seeds is presented in Fig. 3a.

3.7. Measurement of PIMT Methyltransferase Activity

A vapor diffusion assay is performed (12). It involves the transfer of a radiolabeled methyl group from *S*-adenosyl (methyl-^{14}C) methionine ((^{14}C)AdoMet) to the methyl acceptor-isoaspartyl-containing protein ovalbumin.

Methyltransferase assays are performed in 40-μL final volumes. Typically, 12 μL of soluble protein extract (approximately 50 μg protein) is mixed with 4 μL *S*-adenosyl (methyl-^{14}C)methionine, 10 μL of isoAsp-containing ovalbumin solution, and 14 μL of sodium citrate solution. Control reactions are performed by

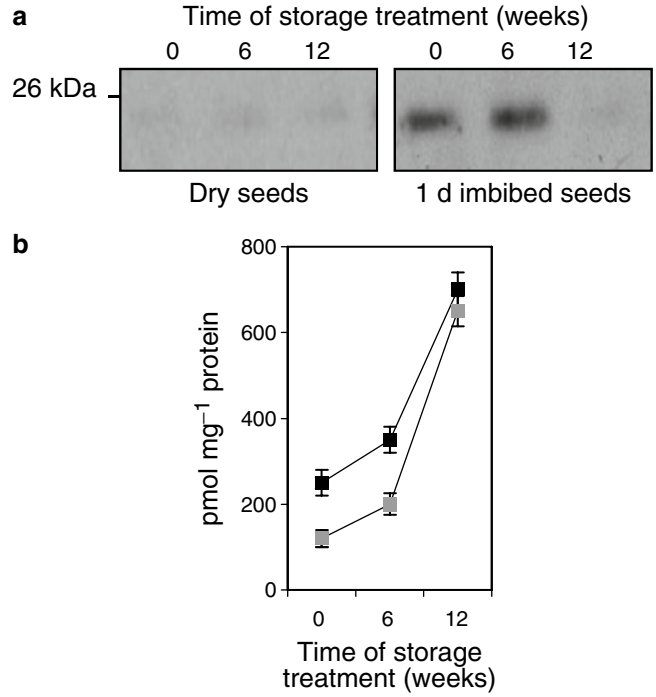

Fig. 3. PIMT1 and quantification of isoaspartyl residues throughout seed storage at 40°C and 32% RH. (**a**) PIMT1 accumulation during the seed storage treatment was estimated in protein extracts (30 μg) from dry seeds and 1-day imbibed seeds using Western blot analysis with polyclonal antibodies raised specifically against PIMT1. (**b**) Quantification of isoaspartyl-containing methyl-accepting substrate was performed on dry seeds (*black squares*) and 1-day imbibed seeds (*gray squares*). Values are from three replicates of 100 mg of seeds each (mean ± SD).

incubating the enzyme extract with buffer alone instead of the ovalbumin protein substrate. The reaction is allowed to proceed at 37°C for 1 h.

1. The reaction is stopped by quenching with 40 μL of stop solution, which results in the hydrolysis of methyl esters to methanol.
2. The samples are placed on ice for 10 min to condense methanol vapors and centrifuged at 16,000 × *g* at 4°C for 10 min.
3. A total of 60 μL of this mixture is immediately spotted on a piece of thick filter paper prefolded in an accordion pleat and placed in the neck of a 20-mL plastic scintillation vial containing 10 mL of ACS II counting fluor and capped. Vials are left at RT for 2 h. During this time, volatile (^{14}C)methanol diffuses into the fluor while the unreacted (^{14}C)AdoMet remains on the filter paper.
4. After removal of the filter paper, radioactivity in the vials is counted by liquid scintillation. Peptide-specific activity is calculated by subtracting the radioactivity from the control (see Note 8).

3.8. Quantification of Damaged Aspartyl and Asparaginyl Residues

3.8.1. Repair Reaction

The ISOQUANT®Isoaspartate Detection Kit uses the enzyme PIMT to specifically detect the presence of isoaspartic acid residues in a target protein (23). PIMT catalyzes the transfer of a methyl group from AdoMet to isoaspartic acid, generating AdoHcy that is detected at 260 nm with HPLC analyses.

1. Methylation reactions are performed in 25-µL final volumes. Typically, 10 µL of total protein extract (25 µg) is incubated for 30 min at 30°C with 5 µL of 5× reaction buffer, 5 µL 100 mM AdoMet, and 5 µL of PIMT enzyme (see Note 9). The positive control is obtained by replacing the protein extract with 75 pmol of isoaspartyl-containing peptide (see Note 10).
2. Reactions are stopped by adding 5 µL of stop solution (acid).
3. Proteins are precipitated by centrifugation at 16,000 × *g* at 4°C for 10 min (proteins precipitate in acidic conditions).
4. Supernatants (30 µL) are transferred to 300-µl vials and kept in the dark at 4°C until HPLC analyses of AdoHcy production. One hundred and fifty microliters of 2 pmol/µL AdoMet are prepared as the standard.

3.8.2. HPLC and UV Detection of AdoHcy

The ISOQUANT®Kit protocol allows the detection of a coproduct of the methylation reaction, AdoHcy (Fig. 1). This small molecule can be quantified by reversed-phase HPLC (RP-HPLC). Results from the test samples are compared to those from an AdoHcy HPLC standard. Methylation reactions and AdoHcy standards are analyzed on a Synergi Hydro-RP column.

The mobile phase A is 50 mM potassium phosphate buffer, pH 6.2 and the mobile phase B is 100% methanol.

The sample method consists of initial parameters that are 10% mobile phase B and 90% mobile phase A at 1 mL/min, detection at 260 nm. After the 12-µL sample injection (see Note 11), a gradient starts to increase the mobile phase B to 30% (70% mobile phase A) over 5 min. Then, the mobile phase B reduces from 30 to 10% (70–90% mobile phase A) over 30 s. The mobile phase B concentration is maintained at 10% (90% mobile phase A) for 7.5 min to equilibrate the column for the next sample.

The cleaning method consists of washing column with 10% mobile phase B, 90% water for 16 min. Methanol is increased to 100% over 5 min, then maintained at 100% for 20 min. Methanol is then decreased to 10% (90% water) over 5 min, and maintained at 10% for 5 min. The column is equilibrated with 10% mobile phase B, 90% mobile phase A for 20 min.

1. Potassium phosphate buffer is prepared and filtered to prevent clogging of HPLC by contaminations.
2. AdoHcy standard, blank (water) and stopped reaction-containing vials, are placed into the autosampler tray cooled to 12°C. To analyze the samples, the sample method described

above is run starting with the injection of two blanks and one AdoHcy standard. The column should be washed (using the cleaning method described above) every ten sample runs followed by blank and standard injections (see Note 12).

3. UV absorbance is monitored online at 260 nm. Quantification of AdoHcy eluted from the column is determined by peak area with reference to a set of AdoHcy standards (see Note 13). An example of the results obtained with protein extracts from dry and 1-day imbibed seeds is presented in Fig. 3b.

4. Notes

1. The appropriate RH should be reached over approximately 1 day.
2. We compared several concentrations of ammonium sulfate in terms of solubilization and protein yields, and we decided to precipitate proteins with 65% ammonium sulfate (which was currently used in our lab). If you want to test other concentrations, we might know that the addition of ammonium sulfate significantly increases the volume of the solution. The amount to add can be determined either from published nomograms or by using an online calculator, for example http://www.encorbio.com/protocols/AM-SO4.htm.
3. A cold room temperature increases markedly the polymerizing time.
4. To facilitate sample loadings, clean the wells by injecting, under pressure, 1× running buffer using a syringe and a fine needle.
5. Beware that there is no leakage of running buffer from the upper chamber to the lower chamber. Otherwise, the migration cannot be made. If this problem appears, begin the assembly again to prevent improper migration.
6. The gel can be stained with Coomassie solution to verify complete transfer of proteins.
7. Hold the membrane with forceps by a corner and drain off the excess of water by applying the opposite corner on an absorbent paper. This will avoid dilution of the substrate.
8. How are counts per minute (cpm) converted into activity (in mol/min)?

   We first need to convert cpm into disintegrations per minute (dpm) taking into account the counting efficiency (Y) (indicated by scintillation counter): $\text{dpm} = \text{cpm} \times \text{Y}$.

   Then, convert into disintegrations per second (dps) and Becquerel (Bq): $\text{dpm} \times 60 = \text{dps} = \text{Bq}$.

Knowing the duration of the enzymatic reaction (60 min in this case), the quantity of Bq, and that (^{14}C)AdoMet has a specific radioactivity (SR) of 185×10^4 Bq/μmol, we can calculate the activity:

$$\frac{\text{Bq}}{\text{SR} \times \text{time}} = \mu\text{mol} \,/\, \text{min}$$

Results can be expressed in mol/mg/min dividing by the quantity of protein used in each experiment.

9. We divided twice the volume of all components to reduce the cost of the experiments.
10. Negative controls may be performed in case there are some difficulties in analyzing the chromatograph (e.g., due to the presence of contaminations) by incubating the sample without PIMT enzyme. Quantification of AdoHcy is measured by subtracting peak area with and without PIMT enzyme for the same sample.
11. Injection of 25 μL can be done if peaks are too small to be quantified; however, this will increase clogging of the column.
12. The filter upstream of the column should be replaced as often as necessary to prevent peak contaminations.
13. Using the sample method described, the AdoHcy peak must be detected approximately 5.3 min after injection.

Acknowledgments

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Chapter 22

Nitric Oxide Methods in Seed Biology

Paul C. Bethke, Igor G.L. Libourel, Jan Vitecek, and Russell L. Jones

Abstract

The ubiquitous signaling molecule nitric oxide (NO) plays an important role in seed biology. Experiments with this biologically important gas require special provisions because NO in aerobic environments is readily converted into other oxides of nitrogen. In this chapter, we describe methods for the application of NO as a gas, and through the use of NO-donor compounds. We included information on the removal or reduction of NO with NO scavengers. Methods for detecting NO using NO-reactive fluorescent probes, and an apparatus incorporating an oxidizer column are also described.

Key words: Nitric oxide donors and scavengers, Seed dormancy and germination, Nitric oxide detection, Application of nitric oxide, Oxidation of nitric oxide, Nitrate and nitrite

1. Introduction

Nitric oxide (NO) is involved in many aspects of plant growth, development, and responses to the environment (1, 2). Compelling evidence points to a central role for NO in the loss of seed dormancy (3). NO is highly reactive, toxic at high concentrations, and unstable. Methods for applying, removing, and detecting NO have been developed (4, 5), but experiments using these methods must take into account the reactivity of NO to prevent artifacts.

1.1. Nitric Oxide Is Unstable in Aerobic Environments

NO is a free radical that is readily converted into other oxides of nitrogen (Fig. 1). High reactivity of NO presents a significant challenge for seed biologists. Many of these nitrogen oxides, including nitrate (NO_3^-), nitrite (NO_2^-), and nitrogen dioxide (NO_2), have potent effects on seeds with respect to dormancy and germination (3). In order to link a particular biological process or enzymatic activity to NO, one must demonstrate that other oxides of nitrogen formed from NO are not the causative agents.

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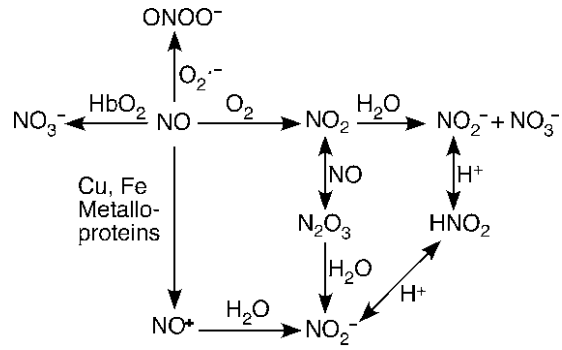

Fig. 1. The reactivity of NO and interconversion of NO with other oxides of nitrogen. NO reacts with oxyhemoglobin (HbO_2), superoxide $O_2^{\cdot-}$, metals or metal-containing proteins, and molecular oxygen (O_2). Additional products resulting from further reactions of NO_2 and NO^+ in aqueous solution are indicated and include nitrate (NO_3^-), nitrite (NO_2^-), and nitrous acid (HNO_2).

The spontaneous reactions that NO undergoes produce different end products under different conditions. In the gas phase, NO interacts with molecular oxygen (O_2) to produce gaseous NO_2. When NO_2 diffuses into an aqueous environment, it is converted into NO_2^- and NO_3^-. In solutions that have a pH of greater than approximately pH 5, reactions of NO with O_2 lead to the preferential accumulation of NO_2^-. At lower pH values, NO_2^- is protonated to nitrous acid (HNO_2), which is in chemical equilibrium with NO_2^-, NO_2, NO, and other oxides of nitrogen. The relative amounts of each depend on the pH of the solution, the presence of metals that can act as catalysts and the redox status of the solution.

1.2. Nitric Oxide Donors

Many pharmacological agents release NO, but relatively few have been used with seeds. Some of the compounds that have been used established roles for NO in seeds with respect to dormancy or germination. The list of donors included in Table 1 indicates that the most commonly used NO donor has been sodium nitroprusside (SNP). SNP has distinct advantages for use as an NO donor, in that it releases NO over many hours and is inexpensive. The disadvantages of SNP are many, and caution is required when interpreting the results of experiments that utilize this compound. The conversion of SNP to NO is promoted by light; therefore, SNP-containing solutions generate NO in the light at a faster rate than in the dark. NO is also produced from SNP within biological tissues, but the biochemistry of this reaction is poorly understood. As with all NO donors, the NO released into solution may participate in nonenzymatic reactions that result in the production of nitrite and/or nitrate. NO produced by SNP is accompanied by cyanide production (4). The effects of cyanide, nitrite, or nitrate on seed germination are well-documented, and care must be taken when

Table 1
Nitric oxide donors and typical concentrations used with seeds

Donor	Compound	Typical concentration	References
SNP	Sodium nitroprusside	100–200 μM	(6, 10, 11, 13–26)
SNAP	S-nitroso-*N*-acetyl-l,l-penicillamine	100–300 μM	(14, 17, 26)
GSNO	S-Nitrosoglutathione		(23)
SIN-1	3-Morpholinosydnonimine	100–1,000 μM	(17)
Nitroglycerine	Glyceryl trinitrate	100–500 μM	(13)
$NO_{(g)}$	Nitric oxide	50 ppm	(7, 16, 27)
NO-saturated water	Nitric oxide		(28)
Acidified nitrite	NO_2^-	25 μM	(10, 11, 15)

using SNP to determine that it is NO, not cyanide, nitrate, or nitrite that cause the response. It is particularly important that appropriate controls are used when SNP is used as the NO donor because cyanide is also volatile. These controls should include the use of KCN, ferrocyanide, ferricyanide, KNO_2, and KNO_3 (6).

SNAP, GSNO, SIN-1, and nitroglycerine are other NO donors that have been used successfully with seeds (Table 1). Each produces NO under suitable conditions, and releases a carrier molecule that may have an effect on its own. For example, GSNO produces glutathione disulfide along with NO (5). Because there is overlap between processes influenced by oxidative stress and NO, suitable controls for glutathione disulfide and glutathione are required. SNAP, like SNP, is photolabile and produces NO when illuminated (4, 5).

$NO_{(g)}$ itself is conceptually the simplest NO donor, but $NO_{(g)}$ has seldom been used in experiments with seeds. An apparatus for delivering defined concentrations of $NO_{(g)}$ is described in Subheadings 2.2–2.4 and 3.4. By delivering gaseous NO in a continuous gas stream, experimental NO levels are kept stable, and gaseous products resulting from NO oxidation are removed. A flow-through setup, therefore, allows for highly controlled, flexible gas application protocols. Because a continuous gas stream washes out volatile endogenous compounds from samples, such as seeds, the application of this approach requires the design of sets of control experiments. Some of the concerns raised by the reactivity of NO apply when using $NO_{(g)}$ as an NO donor, particularly the formation of nitrite (7).

A somewhat simpler method for applying NO directly is to use dilutions of NO-saturated water. Depending on the system, this approach is limited to brief NO exposure periods because of the quick breakdown of NO, especially in the presence of oxygen.

Table 2
Nitric oxide scavengers and typical concentrations used with seeds

Scavenger	Compound	Typical concentration (μM)	References
PTIO	2-Phenyl-4,4,5,5, -tetramethylimidazoline-1 -oxyl-3-oxide	50–200	(11, 12, 28)
c-PTIO	2-(4-Carboxyphenyl)-4,4,5,5 -tetramethylimidazoline- 1-oxyl-3-oxide	50–200	(6, 7, 10, 14–16, 18, 21, 23, 25–27)
TMA-PTIO	3,3,4,4-Tetramethyl-2 -trimethylammoniophenyl-2- imidazoline-3-oxide-1-yloxy	100	(6)
Hemoglobin	In vivo protein		(8)

1.3. Nitric Oxide Scavengers

NO scavengers are important complements to NO donors, and in many cases have provided the best evidence for an involvement of endogenous NO in a particular biological process. The most useful NO scavenging compounds have been PTIO and its derivatives c-PTIO and TMA-PTIO (Table 2). c-PTIO is cell permeable, whereas TMA-PTIO is relatively nonpermeable to cells. An involvement of NO in seed dormancy, for example, was demonstrated when c-PTIO was used as an NO scavenger to strengthen dormancy in a population of *Arabidopsis* seeds (Fig. 2a), but c-PTIO did not inhibit germination of control seeds that were not dormant (Fig. 2b). Nitrite is produced by PTIO, c-PTIO, and TMA-PTIO as part of the NO-scavenging reaction. Hemoglobin is likely to function as an NO scavenger in vivo (8), but hemoglobin application as NO scavenger is complicated by its ability to bind CN.

NO donors and NO scavengers are often used together to demonstrate a specific effect for one or the other compound. Hence, an effect observed upon addition of an NO donor is likely to be an effect of NO if that effect can be prevented by simultaneous application of an NO scavenger. Likewise, an effect of an NO scavenger is more likely to be an effect resulting from NO removal if it can be reversed by simultaneous addition of an NO donor.

1.4. Nitric Oxide Detection

Several methods for detecting and quantifying the NO produced in plants are available. NO detectors based on electrochemical signals, chemiluminescence, photoacoustic effect and electron paramagnetic resonance allow for precise determination of NO, but require specialized equipment that may be prohibitively expensive (9). Alternative approaches suitable for many applications in seed biology include the use of NO-sensitive fluorescent probes and an oxidizer column-based apparatus that uses the Griess reagent to measure oxidized NO (9).

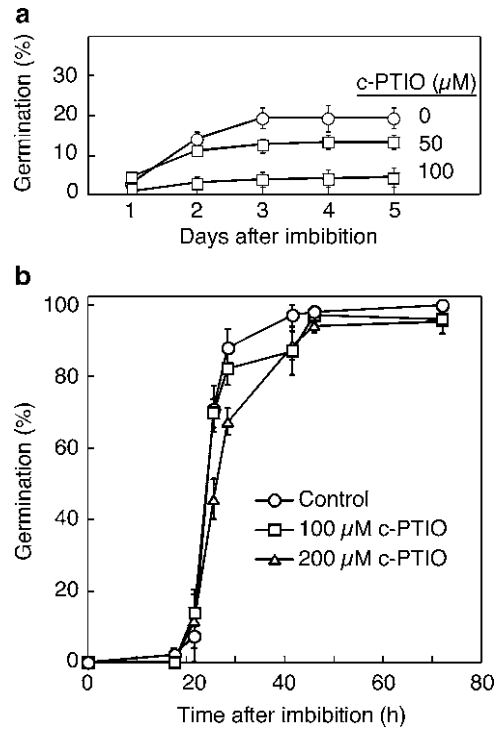

Fig. 2. The effect that the NO scavenger c-PTIO has on dormancy of *Arabidopsis* seeds, modified from Bethke et al. (16). Seeds were imbibed in solutions containing the indicated concentrations of c-PTIO. Seed in (**a**) had weak dormancy and c-PTIO reduced germination by strengthening dormancy. Seeds in (**b**) were not dormant, and c-PTIO did not inhibit germination of these seeds.

Some of the fluorescent compounds that have been used for NO detection in seeds or seed tissues are listed in Table 3. Each of these molecules has a high degree of specificity for NO, yet none have an absolute specificity. This caveat necessitates thoughtful selection of controls and careful interpretation of data in order to avoid misinterpreting fluorescent signals that arise independently of NO. One class of suitable controls includes molecules that are structurally related to the NO-reactive probe but incapable of reacting with NO. Examples include 4-AF as a control for DAF-2 and 1-AQ as a control for DAAQ. An alternative control can be prepared by reacting the NO-reactive probe with NO prior to use, making the fluorescent probe insensitive to NO. Any change in fluorescent signal following this treatment must be ascribed to parameters other than NO. Another useful control is inclusion of an NO scavenger, such as c-PTIO in the labeling solution. In this case, the fluorescence signal should be greatly diminished relative to that of seeds or seed tissues incubated with the fluorescent probe alone.

Direct detection of NO is technically difficult, but detecting the products resulting from NO oxidation is much simpler. The Griess reagent is used to measure nitrite or nitrogen dioxide in

Table 3
Fluorescent probes for the detection of NO

Probe	NO reactive	Compound	Typical concentration (μM)	References
DAF-FM	Yes	4-Amino-5-methylamino-2′,7′-difluorofluorescein	1–10	(10)
DAF-FM DA (cell permeable form of DAF-FM)	Yes	4-Amino-5-methylamino-2′,7′-difluorofluorescein diacetate	5	(11, 12)
DAF-2 DA	Yes	4,5-Diaminofluorescein diacetate	5–20	(26)
4-AF	No. Control for DAF-2 DA	4-Aminofluorescein diacetate	5–20	(26)
DAAQ	Yes	1,2-Diaminoanthraquinone	5–20	(26)
1-AQ	No. Control for DAAQ	1-Aminoanthraquinone	5–20	(26)
DAR-4M	Yes	Diaminorhodamine-4M	5–20	

solution, and has been used extensively as a component of NO detection methods. In most of these methods, NO is first converted into nitrite through enzymatic or nonenzymatic reactions and the resulting nitrite is measured as a change in the absorbance of Griess reagent. An oxidizer column can thus be used to convert the NO released by biological systems into nitrogen dioxide, which is subsequently trapped and quantified in a Griess reagent solution. This approach is easy to implement, highly sensitive and selective (see Note 1). Griess-based detection was used successfully to detect NO in solution as well as gaseous NO produced by nitrate-treated barley aleurone layers (9).

2. Materials

2.1. Solutions

1. SNP stock solution: 10 mM SNP. Dissolve 29.8 mg of SNP (MW = 298) in 10 mL of water. Dilute with distilled H_2O or buffer to produce the required final concentration. To be freshly prepared immediately before use and protected from the light to prevent photolysis. (Avoid breathing SNP vapors since they contain low concentrations of cyanide).
2. c-PTIO stock solution: 50 mM c-PTIO. Dissolve 25 mg of c-PTIO in 1.58 mL distilled water. Divide into aliquots and

freeze at –20°C. Frozen stocks are stable for at least 1 year, but it is important to avoid repeated freezing and thawing. Make working strength solutions immediately before use and discard unused portions.

3. DAF-FM solution: 5.5 mM DAF-FM in DMSO. Aliquot and freeze until use. Dilute an aliquot of DAF-FM 1:100 with water or fresh incubation medium for a working strength solution.
4. Griess reagent: 4% sulfanilamide, 0.2% *N*-(1-naphthyl)-ethylenediamine, 10% H_3PO_4. The Griess reagent is available commercially (LabChem Inc., Pittsburgh, PA), but can be prepared from analytical grade chemicals. Dissolve sulfanilamide in about half of the final volume of distilled water containing the appropriate amount of H_3PO_4. Dissolve *N*-(1-naphthyl)-ethylenediamine separately in a small amount of distilled water with intensive shaking. Mix both solutions and bring to the desired volume with distilled water. Filter the final solution through analytical-grade filter paper. The reagent is stable for several months if it is protected from light and refrigerated. It should be discarded when the color begins to turn brown.

2.2. NO-Containing Compressed Gas Cylinders and Related Equipment

1. Compressed gas cylinders containing mixtures of NO are available commercially (Praxair, Los Angeles, CA). The NO concentration requested from the supplier should be governed by the desired application. Since N_2 is used as the carrier gas for NO, the maximum concentration available under normoxic conditions is 80% of the cylinder concentration after mixing with pure O_2 (see Note 2).
2. An NO concentration greater than 25 ppm is considered a health hazard. It is advisable, therefore, to minimize the potential risk of exposure by using compressed gas tanks with relatively low NO concentrations. We used NO at 212 ppm in nitrogen.
3. Specialty gas regulators for corrosive gases must be used for NO and O_2. (stainless steel, Fisher Scientific, Pittsburgh, PA).
4. Tubing, valves, and tubing connectors should be suitable for NO application and should not contain brass. Kalrez® and Viton® or other Teflon materials are suitable (see application notes of NO gas supplier). Stainless steel is acceptable, but the use of metal should be avoided when possible.

2.3. Regulating Gas Flow Rates

Gas flow rates need to be controlled and measured. When constant flow rates are being used and less precision is acceptable, gas flow can be controlled with simple metering valves (Swagelok, Solon, OH). High precision and flexible application regimes require the use of mass flow controllers (MFCs) (see below and Fig. 3). The pressure between gas regulators and flow regulators is kept

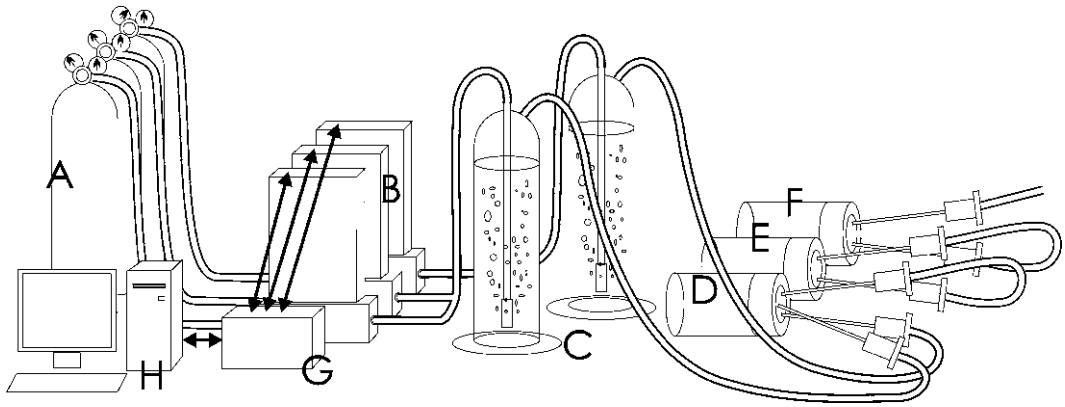

Fig. 3. Diagram of the NO application setup, modified from Libourel et al. (7). O_2, N_2, and NO are supplied using compressed gas cylinders (A). The gas flows are controlled using mass flow controllers (MFCs) (B) and then lead through a sparger filled with 0.5 M KOH (C) for humidification and scrubbing of nitrite. O_2 and NO are kept separate and are mixed just upstream of the samples in a mixing vessel (D). To fine-tune gas humidity, the gas mixture is blown over an additional distilled water-filled, capped, serum vial (E) placed before the sample vial (F). The MFCs are hooked-up to an interface unit (G) that serves as power supply for the MFCs and facilitates the communication between a computer (H) and the MFCs.

at ~30 psi as flow meters and MFCs require approximately this pressure difference for optimal performance. Gas lines and connectors must be adequate for this pressure, such as Swagelok PFA tubing and Swagelok connectors.

2.3.1. MFCs and MFC Control Units

1. MFC manufacturers include Brooks Instrument (Hatfield, PA), MKS Instruments (Andover, MA), Aera Corp. (Austin, TX), Porter (Hatfield, PA), STEC/Horiba (Austin, TX), and Sierra Instruments Inc. (Monterey, CA). In our experimental setup, we used Tylan FC-280 AV (see Note 3).
2. MFC manufacturers produce control units that include a stable power supply, dials to control flow rates, and displays indicating flow through the MFCs. Advanced controllers also include a communication interface that allows for software-controlled operation and data logging (see Notes 4 and 5).
3. Custom-built computer-controlled set-point controllers can be assembled. We used an RS232-controlled analog to digital converter (WTADC-M) and two digital to analog converter (WTDAC-M) cards (Weeder Technologies, Walton Beach, FL) for monitoring and set-point control, respectively (see Note 6). Power for the MFCs was supplied by a Sola 3030-15T power supply (Mouser Electronics, Mansfield, TX).

2.4. Device Software

1. Most control units support simple serial text-based protocols, such as RS232 and its successors, but more advanced (digital) MFC units communicate with computers directly (see Note 7).

2.5. The NO Oxidizer Column

1. Glass tubing (1.5 cm diameter, length ~30 cm), glass wool, rubber stoppers, large bore injection needles, and Pyrex glass beads of 3 mm diameter.
2. Bead-coating solution prepared by dissolving 4 g of potassium dichromate and 1.5 mL of concentrated sulfuric acid (98%) in 25 mL distilled water.

2.6. Additional Equipment Required for an Oxidizer Column-Based Apparatus

1. Suitable plastic tubing for connecting the air supply to the spargers and the oxidizer column (Tygon, PVC, or silicone, internal diameter 1–3 mm).
2. Equipment capable of supplying air at a constant flow rate of 40 mL/min and a pressure of 10–20 kPa is required. A computer-operated flow-through system as described in Subheading 3.4 is ideal. Alternatively, a gas pump together with a pressure gauge and flow meter can be used (see Note 8).
3. NO gas for testing of the oxidizer column-based detector (see Subheading 2.2).
4. Gas washing bottle with a fritted glass sparger containing water to humidify air (Fig. 4a).
5. A sample chamber equipped with gas inlet and outlet having a volume as low as reasonably achievable in order to minimize the amount of gas required for complete flushing. The configuration of the chamber should allow passing of the air along the whole surface of a solid sample or the inlet should reach under the liquid surface in the case of NO sampling from a liquid.

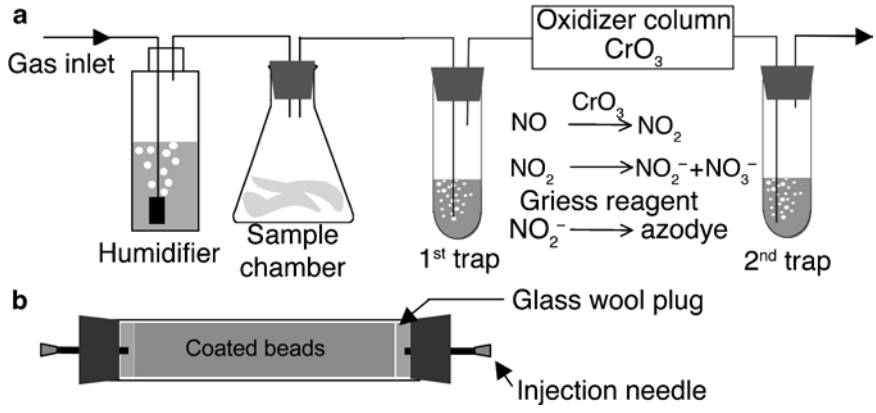

Fig. 4. Oxidizer column-based apparatus for measuring NO release. (**a**) Schematic representation of the setup and the underlying chemistry: Nitrous acid is captured in the first trap in the form of nitrite, whereas NO passes through the oxidizer column, where it is converted to nitrogen dioxide and captured in the second trap. Both traps contain Griess reagent to convert nitrite formed to an intensely pink colored azo dye. (**b**) Scheme of the oxidizer column: Chromium trioxide coated beads are packed in a glass tube stoppered on both ends with a glass wool plug and a rubber bung containing an injection needle.

6. Two small gas traps that contain the Griess reagent detection solution. These are made from glass test tubes (~15 mL), PEEK tubing (0.5-mm internal diameter), and two-holed rubber stoppers (Fig. 4a).

3. Methods

3.1. Imbibing Seeds with NO Donors and NO Scavengers

Germination assays are commonly used to assess the effect of NO donors and NO scavengers on seed dormancy. Seeds are imbibed on filter paper or on agarose pads.

1. One or two layers of filter paper (e.g., Whatman A2) are arranged in the bottom of a Petri dish and are moistened with a measured amount of NO-donor- or NO-scavenger-containing solution. A film of water should be visible on the surface of the filter paper, but excess moisture is avoided.
2. Seeds are placed onto the moist filter paper.
3. Evaporation is prevented by using dishes with tightly fitted lids, or by wrapping with Parafilm. This is essential to prevent changes in the concentration of applied compound.
4. Dishes are wrapped in aluminum foil or placed in a light-tight incubator for imbibition and germination in the dark. Alternatively, dishes may be placed under lights, taking care to avoid elevated temperatures by using low-temperature fluorescent or photodiode illumination where possible.
5. Pads of solidified agarose are used as an alternative to moist paper for applying compounds. Agarose (~1%) is dissolved in distilled water by heating to boiling in a microwave oven with occasional swirling. NO donors or NO scavengers are added to the dissolved agarose when it has cooled to near its melting point. The molten agarose is then pipetted into dishes using a plastic serological pipette and is allowed to solidify. Seeds can be sown directly on the cool agarose, or on nylon filter disks placed on top of the agarose. The latter are useful for removing seeds from an original treatment compound and transferring them, while still on the nylon membrane, to a second pad containing a fresh treatment solution or an alternative solution.

3.2. Determining NO in Solution Using DAF-FM

Fluorescent probes can be used to test for the presence of NO in solution. A sample of the solution is added to a buffered medium containing an NO-reactive fluorescent probe, and after a short incubation period, the fluorescent signal is measured with a fluorometer or fluorescence microplate reader. DAF-FM was used in this way to demonstrate NO production in media from barley aleurone layers (10) (see Notes 9 and 10).

1. Combine 280 μL of filter sterilized (0.2-μm pore size) incubation medium with 35 μL DAF-FM working solution and 35 μL distilled water, SNP (1 mM), or c-PTIO (1 mM). Samples with SNP or c-PTIO serve as positive and negative controls for the reaction, respectively.
2. Incubate at room temperature until the reaction between NO in solution and DAF-FM goes to completion, approximately 30 min.
3. Combine 100 μL of the DAF-FM assay mixture with 2 mL of 50 mM Hepes, pH 7.75.
4. Quantify fluorescence at an excitation wavelength of 495 nm and an emission wavelength of 515 nm.

3.3. NO Production by Seeds and Seed Tissues Using Fluorescent Probes

NO-reactive fluorescent probes can also be used to demonstrate NO production by seeds or seed tissues by observing increases in fluorescence from seeds or tissues rather than from the solution. In general, this has required extended incubation times of 1 h (10) to 2 days (11, 12). During this period, a slow increase in the fluorescent signal is observed. Long incubation times make it difficult to distinguish between uptake of the nonfluorescent probe by the tissue followed by conversion to the fluorescent molecule within the tissue, or uptake of the fluorescent molecule after it has reacted with NO in the medium. Likewise, using intact seeds, it has not been possible to identify specific regions of the seed that are involved in NO production because it is difficult to distinguish between sites of NO production and sites of fluorescent reporter molecule accumulation. Often the preferred sites of intracellular accumulation for the fluorescent molecule are not known, but specific localization to vacuoles or other organelles is common.

3.4. Experimental Setup for Regulated Delivery of NO Gas

1. The equipment setup is illustrated in Fig. 3 and is as described previously (7).
2. Position spargers in the gas stream after the MFCs to humidify the application gas. This prevents dehydration of the samples (see Note 11). Because NO reacts with O_2 particularly rapidly under aqueous conditions, the NO-carrying gas should be humidified separately from the O_2 containing gas. The humidified gas streams should be mixed just prior to the sample chamber to limit NO degradation.
3. To minimize NO degradation the volume of the mixing vessel is small (5 mL), which forces a quick gas replacement rate.
4. Place a dummy sample vial with KOH in the gas stream just prior to the first sample bottle to scrub nitrite formed in the mixing chamber and catch liquid carry over from the spargers.
5. Place samples on 3 mL, 1% agarose pads (Subheading 3.1 and Fig. 3) inside the sample vials, cap the vials and place them in

the gas stream (see Note 12). Because the application gas has 100% humidity, application of NO can be maintained for several days without affecting the volume of the agarose pads. The NO-containing gas stream is exhausted in a fume hood to prevent potentially harmful exposure of laboratory personnel.

6. Adjust the flow rate of the application gas to as high as reasonably possible without pressurizing the spargers or causing excessive liquid carry over. We commonly use flow rates between 50 and 100 mL/min.

3.5. NO Detection with an Oxidizer Column-Based Apparatus

1. Thoroughly mix 100 mL of 3-mm Pyrex glass beads with 4 mL of the bead-coating solution in a broad glass beaker. Place the beaker into a dry thermostat at 80°C and stir beads occasionally until the yellow color of the wet beads starts to change to brick red. Stir continuously until the color change is complete. Leave the beads in the thermostat for about 5 min to finish drying. Some batches of dichromate may produce a brownish color instead of brick red, but this does not affect the performance of the oxidizer column.
2. Pack dried, coated beads into a glass tube and stopper both ends with a glass wool plug and a rubber bung. Syringe needles piercing the stoppers are used as input or output for gas (Fig.4b). Seal the gas input and output with plastic wrap to prevent absorption of moisture from the air. Oxidizer columns are stable for several weeks.
3. Connect components to the oxidizer column as indicated in Fig. 4a (see Notes 13 and 14).
4. Supply air from compressed gas tanks using computer-operated MFCs (Subheadings 2.2, 2.3 and 3.4) or pump air through the setup using a suitable air pump (see Note 8).
5. Set the airflow rate to 40 mL/min and purge the system with at least 200 mL plus ten sample chamber volumes. Stop the airflow.
6. Add 1 mL of the Griess reagent to both traps.
7. Put the sample into the sample chamber and set the airflow to 40 mL/min. Sampling needs to be carried out until there is a visible color change in the second trap (typically 10–60 min).
8. Remove the sample chamber and purge the system with at least 100 mL of pure, moist air at 40 mL/min in order to flush nitrogen oxides from the dead volume of the apparatus.
9. Determine the absorbance of the Griess reagent in the traps at 545 nm 10 min after column flushing. The response is calibrated by preparing a standard curve of nitrite in the Griess reagent.
10. Determine the background response of the detector system using the procedure in steps 6–9 above using an empty sample

chamber and the same sampling time. This background response is subtracted from that obtained for the sample.

11. Change the column when 2/3 of the beads have become hydrated, as indicated by a change in color from brick red to yellow (see Note 15).
12. To check recovery rate of the detector use NO gas from a tank delivered directly to the sample chamber by an MFC (see Subheadings 2.2, 2.3, and 3.4). Combine NO with the airflow so that the final concentration of NO is less than 1 ppm (see Note 1). Proceed according to steps 6–10 above and calculate the recovery rate.

4. Notes

1. The oxidizer column detector has a detection limit for NO of 0.1–0.2 nmol/h (equivalent to 1–2 ppb of NO when sampling at a flow rate of 40 mL/min at 25°C for 1 h). The recovery rate (more than 90%) has been found to be constant at NO concentrations below 1 ppm (equivalent to an NO release rate of 100 nmol/h under the conditions specified above). There is some variation of recovery rate depending on column batches. Nitrous oxide (N_2O) also gives a positive signal but only at very high concentrations (>1‰) well beyond the levels of N_2O produced by biological systems. Volatile nitrogen-containing compounds such as hydrogen azide, hydrogen cyanide, and ammonia, do not show a false positive signal with the oxidizer column.
2. NO cylinder concentrations higher than needed are not advised because NO degrades at room temperature via a disproportionation reaction, where $4NO \rightarrow N_2O_3 + N_2O$. This reaction is quadratically dependent on concentration and can reduce the shelf life of the NO cylinder significantly.
3. MFCs are devices that actively regulate gas flow while compensating for changes in gas pressure. MFCs are constructed with an internal flow meter, and they constantly adjust an internal valve to produce a set flow rate.
4. A cautionary note: Control units and MFCs of different manufacturers may not be compatible.
5. The quality and feature richness of MFCs varies considerably. Basic models must be calibrated for a specific gas and are controlled by a simple set-point voltage or current loop. Advanced models are calibrated for usage with any gas, have a digital communication interface and can directly be controlled with RS232 or DeviceNet®.

6. Data acquisition programing interfaces, such as Labview (National Instruments, Austin, TX) can use RS232 devices, and this makes programing relatively straightforward for simple applications.
7. Weeder Technologies provide free data acquisition and control software (ModCom) for use with their hardware. Many current PCs ship without serial RS232 ports, but a USB to serial adapter cable usually works.
8. As an alternative to MFCs, an air pump meeting the specifications in Subheading 3.5 can be used to supply air to the system. A "T"-pipe is connected to the pump to split the airflow between the flow-through system and an outlet tube. A pressure meter is inserted immediately after the T-junction on the flow-through system branch. A pinchcock or needle valve on the outlet tube is used to set the pressure. A constant flow rate (±10%) monitored at the end of the flow-through system can be achieved by maintaining appropriate pressure (10–20 kPa). The flow-through NO detector can be tested by pumping air through a rubber-stoppered flask containing a defined amount of NO at concentrations <1 ppm. This test flask is placed into the system instead of the sample chamber and airflow equal to at least ten times of the volume of the flask plus the 100 mL necessary for flushing the column is pumped through the system.
9. Because the fluorescence of DAF-FM, like that of DAF-2, is quenched at acidic pH, the assay medium is buffered so that the pH is >pH 7 when measuring fluorescence.
10. The formation of a fluorescent product from an NO-reactive probe depends on both the rate of NO production, and on the rate that NO reacts with the fluorescent probe relative to the rate that it reacts with endogenous compounds in the sample. Treatments or conditions that change the amount of NO-reactive, endogenous compounds will change the apparent rate of NO production if the endogenous compounds react with NO at a rate comparable to that of the fluorescent probe. Care must be taken to make sure that this change is not identified as a change in NO production.
11. The design of most MFCs necessitates the use of dry gas in order to obtain accurate flow rates. Because of this, MFCs should always be placed upstream of gas humidification. We use 500-mL Pyrex brand (Fisher Scientific, Pittsburgh, PA) spargers for humidification.
12. The sample chambers (5-mL serum vials, capped with 20-mm straight plug butyl stoppers, Wheaton, Millville, NJ) and the diameter of connecting tubing is kept small, to minimize the dwell time of the application gas mixture.

13. The first Griess trap before the oxidizer column accumulates nitrous acid (HNO_2) emitted from the biological system. The second trap detects nitrogen dioxide (NO_2) formed by oxidation of NO in the oxidizer column.
14. Components of the flow-through system can be connected easily using commonly available laboratory supplies. In order to connect the oxidizer column, an injection needle adaptor is required. A small plastic pipette tip cut on both ends so that it can fit tightly to the injection needle as well as to the plastic tubing that can meet this demand. It is advisable to check gas-tightness of all connections using a pressure meter or detergent solution (formation of bubbles indicates a leak).
15. The column shows a constant recovery rate for NO until more than 2/3 of the column packing is hydrated. This allows for about 3 h of measurement. Using precolumns with molecular sieves or spargers with a saturated solution of $CaCl_2$ to remove water vapor from the gas phase is not effective in extending column life, but rather causes failure of NO detection.

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Part VIII

Bioinformatic Resources

Chapter 23

Seed Bioinformatics

George W. Bassel, Michael J. Holdsworth, and Nicholas J. Provart

Abstract

Analysis of gene expression data sets is a potent tool for gene function prediction, *cis*-element discovery, and hypothesis generation for the model plant *Arabidopsis thaliana*, and more recently for other agriculturally relevant species. In the case of *Arabidopsis thaliana*, experiments conducted by individual researchers to document its transcriptome have led to large numbers of data sets being made publicly available for data mining by the so-called "electronic northerns," co-expression analysis and other methods. Given that approximately 50% of the genes in *Arabidopsis* have no function ascribed to them by "conventional" homology searches, and that only around 10% of the genes have had their function experimentally determined in the laboratory, these analyses can accelerate the identification of potential gene function at the click of a mouse. This chapter covers the use of bioinformatic data mining tools available at the Bio-Array Resource (http://www.bar.utoronto.ca) and elsewhere for hypothesis generation in the context of seed biology.

Key words: Gene expression analysis, Co-expression analysis, Microarrays, Data mining, Gene function prediction, *Arabidopsis thaliana*, Seed biology

1. Introduction

This chapter touches on four aspects of online gene expression analysis (1) generating "electronic northerns"; (2) the *e*lectronic *F*luorescent *P*ictograph (eFP) Browser to graphically examine the expression pattern of one or several genes across many seed data sets; (3) Expression Angler to identify co-expressed genes across all seed data sets; and (4) TAGGIT Gene Ontology classification. Various other seed bioinformatic resources are discussed in brief.

The concept of "guilt-by-association" is predicated on the assumption that genes involved in the same pathway will share similar gene expression patterns. This method for assigning gene

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function to uncharacterized genes or genes with unknown function is well-recognized in yeast, and has also been extended to multicellular organisms, such as mouse and human (1–3). In the case of Arabidopsis, thousands of gene expression data sets have been generated by individual research laboratories and by the AtGenExpress Consortium, including the Developmental Map data set (4) and Global Stress data set (5). These have been archived in public microarray databases, such as the Gene Expression Omnibus (GEO) (6), ArrayExpress (7), NASCArrays (8), The Arabidopsis Information Resource (TAIR) (9), Genevestigator (10), and the Bio-Array Resource (BAR) (11). These sites have query pages for exploring the data sets to retrieve the expression levels for a desired gene under specific treatments or in given tissues. In addition, several Web-based co-expression analysis tools for identifying genes that are co-expressed with a researcher's favourite gene have been developed in the past couple of years. Such programs include the Arabidopsis Co-expression Tool (ACT) (12), ATTED-II (13), AthCoR (14), and Expression Angler (11). The specific features of each of these programs are described thoroughly by Aoki et al., who also discuss the topic of co-expression networks in plants (15).

More computational studies that use large-scale expression data sets to predict gene function and regulatory modules, particularly within the context of curated lists of genes, have also recently been conducted. Examples of studies using this approach in Arabidopsis include those targeting the genes for isoprenoid biosynthesis (16) or for the cytochrome P450 superfamily (17). Such computational studies are ignored here in favour of focusing on the use of Web-based methods for identifying expression patterns and co-expressed sets of genes for one's gene or genes of interest, especially in the context of seed biology.

2. Materials

The methods described in this chapter simply require access to a computer with a fast Internet connection and a Web browser. Data from such tools may be downloaded as text-based files that can be opened with a spreadsheet program, such as Microsoft Excel, for further processing. Gene lists for querying the database in question, in the sense of a series of Arabidopsis Genome Initiative (AGI) identifiers, such as At3g24560, can be copied from a spreadsheet: each gene identifier should be in its own cell and on a different row of the spreadsheet. Alternately, the list may be copied from a word processor or text editor, again such that each AGI identifier is on its own line.

3. Methods

3.1. Electronic Northern Analysis: eFP Browser

The electronic Fluorescent Pictograph (eFP) Browser is an online resource for the analysis and visualization of gene expression data (18). With other tools, a two-dimensional matrix of colours representing different levels of transcript abundance – the so-called heatmap – has been used to visualize the expression levels of genes across many experiments. In contrast, the eFP Browser contains pictographic representations of the organism, tissue, cell type, or condition examined for a given set of experiments, along with notes regarding how the experiments were conducted. Visual representations assist user interpretation of these data over the coloured squares used in "customary" heatmaps:

1. Point a Web browser to http://www.bar.utoronto.ca and select the "Arabidopsis eFP Browser" link on the BAR homepage.
2. Select the "Data Source" to visualize different collections of microarray data. The default is the Arabidopsis Developmental Map from Detlef Weigel's group in Tübingen, Germany (4). For seed biology, the "Seed" Data Source is most relevant. This comprises a collection of 151 microarray data sets generated using the Affymetrix ATH1 platform, from 53 experiments related primarily to seed dormancy, after-ripening (AR), and the chemical inhibition of germination (19).
3. For a given gene of interest, enter the AGI identifier in the "Primary AGI ID" field. The Mode may be left in its default "Absolute" setting. A discussion of the different Modes may be found in Subheading 4 (see Note 1).
4. Click GO.
5. Standard deviation filtering may be clicked in the output screen to mask samples with large variability between replicates, if these are present. These samples are "masked" with a grey colour in the eFP Browser output.

   The correct interpretation of the eFP Browser output requires examination of the colour scale denoting the normalized absolute signal intensities from the microarray data sets. The colour scale in the example output image shown in Fig. 1 is found in the top right of the output image for the Seed eFP output and varies from a lighter yellow colour (negligible expression level) to a darker red colour (higher expression level). In the case of the GCOS/MAS5.0 normalization method used for public microarray data in the BAR, a signal value below 20 is background; genes with a maximum signal intensity lower than this value are not significantly expressed. *CYP707A2* in Fig. 1 has a maximum value of 730 in the Seed

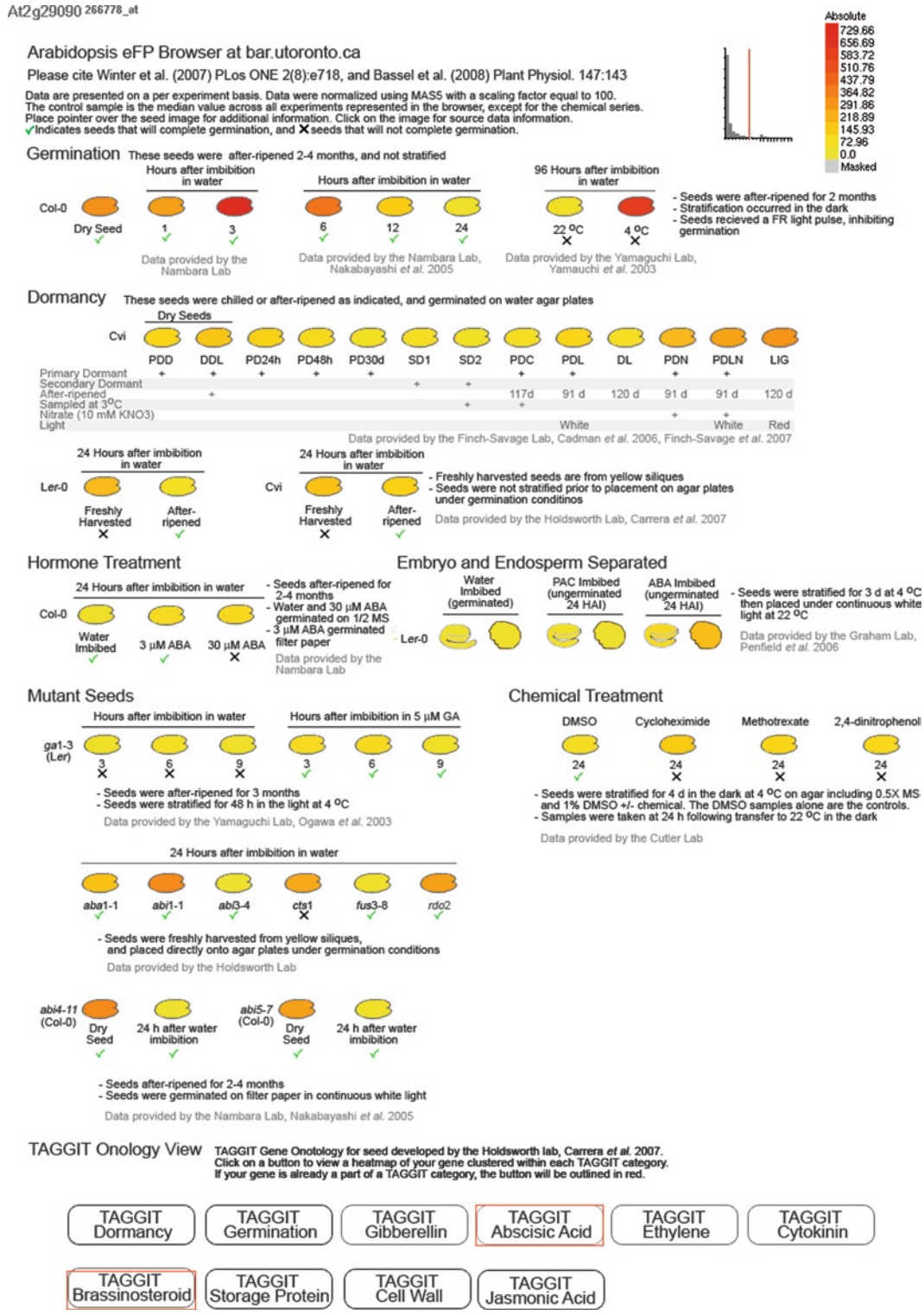
At2g29090 266778_at
Arabidopsis eFP Browser at bar.utoronto.ca
Please cite Winter et al. (2007) PLos ONE 2(8):e718, and Bassel et al. (2008) Plant Physiol. 147:143
Data are presented on a per experiment basis. Data were normalized using MAS5 with a scaling factor equal to 100.
The control sample is the median value across all experiments represented in the browser, except for the chemical series.
Place pointer over the seed image for additional information. Click on the image for source data information.
✓Indicates seeds that will complete germination, and ✗seeds that will not complete germination.
Absolute
729.66
656.69
583.72
510.76
437.79
364.82
291.86
218.89
145.93
72.96
0.0
Masked
Germination
These seeds were after-ripened 2-4 months, and not stratified
Hours after imbibition in water
Hours after imbibition in water
96 Hours after imbibition in water
Col-0
Dry Seed
1
3
6
12
24
22 °C
4 °C
- Seeds were after-ripened for 2 months
- Stratification occurred in the dark
- Seeds recieved a FR light pulse, inhibiting germination
Data provided by the Nambara Lab
Data provided by the Nambara Lab, Nakabayashi et al. 2005
Data provided by the Yamaguchi Lab, Yamauchi et al. 2003
Dormancy
These seeds were chilled or after-ripened as indicated, and germinated on water agar plates
Dry Seeds
Cvi
PDD
DDL
PD24h
PD48h
PD30d
SD1
SD2
PDC
PDL
DL
PDN
PDLN
LIG
Primary Dormant
Secondary Dormant
After-ripened
Sampled at 3°C
Nitrate (10 mM KNO3)
Light
117d
91 d
120 d
91 d
91 d
120 d
White
White
Red
Data provided by the Finch-Savage Lab, Cadman et al. 2006, Finch-Savage et al. 2007
24 Hours after imbibition in water
Ler-0
Freshly Harvested
After-ripened
Cvi
- Freshly harvested seeds are from yellow siliques
- Seeds were not stratified prior to placement on agar plates under germination conditinos
Data provided by the Holdsworth Lab, Carrera et al. 2007
Hormone Treatment
24 Hours after imbibition in water
Col-0
Water Imbibed
3 µM ABA
30 µM ABA
- Seeds after-ripened for 2-4 months
- Water and 30 µM ABA germinated on 1/2 MS
- 3 µM ABA germinated filter paper
Data provided by the Nambara Lab
Embryo and Endosperm Separated
Water Imbibed (germinated)
PAC Imbibed (ungerminated 24 HAI)
ABA Imbibed (ungerminated 24 HAI)
Ler-0
- Seeds were stratified for 3 d at 4 °C then placed under continuous white light at 22 °C
Data provided by the Graham Lab, Penfield et al. 2006
Mutant Seeds
Hours after imbibition in water
Hours after imbibition in 5 µM GA
ga1-3 (Ler)
3
6
9
3
6
9
- Seeds were after-ripened for 3 months
- Seeds were stratified for 48 h in the light at 4 °C
Data provided by the Yamaguchi Lab, Ogawa et al. 2003
24 Hours after imbibition in water
aba1-1
abi1-1
abi3-4
cts1
fus3-8
rdo2
- Seeds were freshly harvested from yellow siliques, and placed directly onto agar plates under germination conditions
Data provided by the Holdsworth Lab
abi4-11 (Col-0)
Dry Seed
24 h after water imbibition
abi5-7 (Col-0)
Dry Seed
24 h after water imbibition
- Seeds after-ripened for 2-4 months
- Seeds were germinated on filter paper in continuous white light
Data provided by the Nambara Lab, Nakabayashi et al. 2005
Chemical Treatment
DMSO
Cycloheximide
Methotrexate
2,4-dinitrophenol
24
24
24
24
- Seeds were stratified for 4 d in the dark at 4 °C on agar including 0.5X MS and 1% DMSO +/- chemical. The DMSO samples alone are the controls.
- Samples were taken at 24 h following transfer to 22 °C in the dark
Data provided by the Cutler Lab
TAGGIT Onology View
TAGGIT Gene Onotology for seed developed by the Holdsworth lab, Carrera et al. 2007.
Click on a button to view a heatmap of your gene clustered within each TAGGIT category.
If your gene is already a part of a TAGGIT category, the button will be outlined in red.
TAGGIT Dormancy
TAGGIT Germination
TAGGIT Gibberellin
TAGGIT Abscisic Acid
TAGGIT Ethylene
TAGGIT Cytokinin
TAGGIT Brassinosteroid
TAGGIT Storage Protein
TAGGIT Cell Wall
TAGGIT Jasmonic Acid

Fig. 1. eFP Browser output using the gene encoding the ABA-degrading enzyme CYP707A2 (At2g29090) using the "Seed" data source. Gene expression increases during early imbibition, as seen by the *darker shading* during that stage.

data set, permitting meaningful interpretation of these data. Where this expression level falls relative to the distribution of the average expression levels across all genes is denoted by the red line on the small histogram on the output image, with the average expression level distribution of all other genes indicated by the grey bar graph. The small ticks denote steps of 100 units while the larger ticks denote steps of 1,000 expression units: clicking on this histogram will enlarge it. In all of the compendia in the eFP Browser, roughly 50% of the genes are expressed at 100 expression units or lower.

By placing the mouse pointer over a given sample, the signal intensity and standard deviation for each sample can be seen in a box, which appears beside the pointer. Clicking on the sample links to the original data source, typically from NASCArrays or GEO, but occasionally from the journal itself or the lab's Web site.

6. Once an AGI ID has been entered, the user can view other data set compendia by changing the option in the "Data Source" pulldown menu.

3.2. Electronic Northern Analysis: Expression Browser

The Expression Brower can be considered as a bulk version of the eFP Browser, in that it accepts multiple gene identifiers rather than one AGI identifier at a time. The Expression Browser output for gene expression is a heatmap matrix, where the rows are indexed by the AGI identifiers and the columns specify the samples from which the data were generated. Across the top of the heatmap are multiple fields with different colours representing information about the sample, including tissue type, whether the sample is a treatment or control, if the data are from a mutant genotype, the relative time scale for time-series experiments, and the age of the plant from which the samples were taken (not relevant for the Seed data set).

In addition to provide information on transcript levels, the Expression Browser also hierarchically clusters the rows in order to group AGI IDs with similar patterns of gene expression across samples, and furthermore displays both documented and predicted interactions between the protein products of the genes in question. These data are displayed to the right of the heatmap as arcs between two gene identifiers.

1. Navigate to http://www.bar.utoronto.ca and click the "e-Northerns with Expression Browser" link on the BAR homepage.
2. Choose the category of data sets you wish to query. Only one category may be selected at a time.
3. Data sets within the data category may be sub-selected in the "Research Area" window. Multiple areas may be selected at once by holding down the Ctrl key. Not clicking in this area selects all experiments.

4. Growth stages, tissue types, and time points may be sub-selected in a similar fashion to step 3.
5. The default output option is the "Average of replicate treatments relative to average of appropriate control". This setting is recommended. The second option "Average of replicate treatments" is also useful when considering absolute expression levels.
6. A list of AGI IDs may then be copied from a spreadsheet program into the appropriate text field below. Each AGI ID must be on its own line.
7. Click Submit.
8. On the output page, links to multiple options for displaying the query results are then displayed. The two options in bold represent the results that have undergone clustering, and are recommended. The first of the two links represents the raw ratio measurements for the genes. These ratios will range from 0 to 1 for genes that are downregulated relative to levels from a control set of experiments to greater than one, where the genes are upregulated. (In the case of the Developmental Map, Tissue Specific, and the Seed series, the control for each gene is its median expression level across all the samples displayed; in the case of abiotic and biotic treatments, the control level for a gene is the level in the mock-treated or untreated plants). In the second output link, the data have been $\log_2$ transformed. This is useful for highlighting differences between data points more clearly than the absolute values.

   Scrolling the mouse over the heatmap displays information about that particular sample in fields above the heatmap. The "Description" field provides a text description of the sample. Clicking on a given column in the heatmap provides a link to the original data. The maximum colour scale for the heatmap can be adjusted in the textbox beside the "Max" button. This can aid in visualizing differences between genes expressed at levels lower than the pre-computed colour-scale maximum. There are other click options (see Note 3).

   To the right of the heatmap is a tree-like graphic representing the way in which the data have been hierarchically clustered. In Fig. 2, two genes of the ethylene signalling pathway, *EIN3* and *CTR1*, show the most similar expression responses: the Pearson correlation coefficient, which can be determined from the small scale above the tree, is around 0.8 (a value of 1 indicates identical responses between the two genes, a value of 0 is no similarity in response and a value of –1 indicates that the responses are opposite). Some caution in interpreting the meaning of these results is advised (see Note 2).

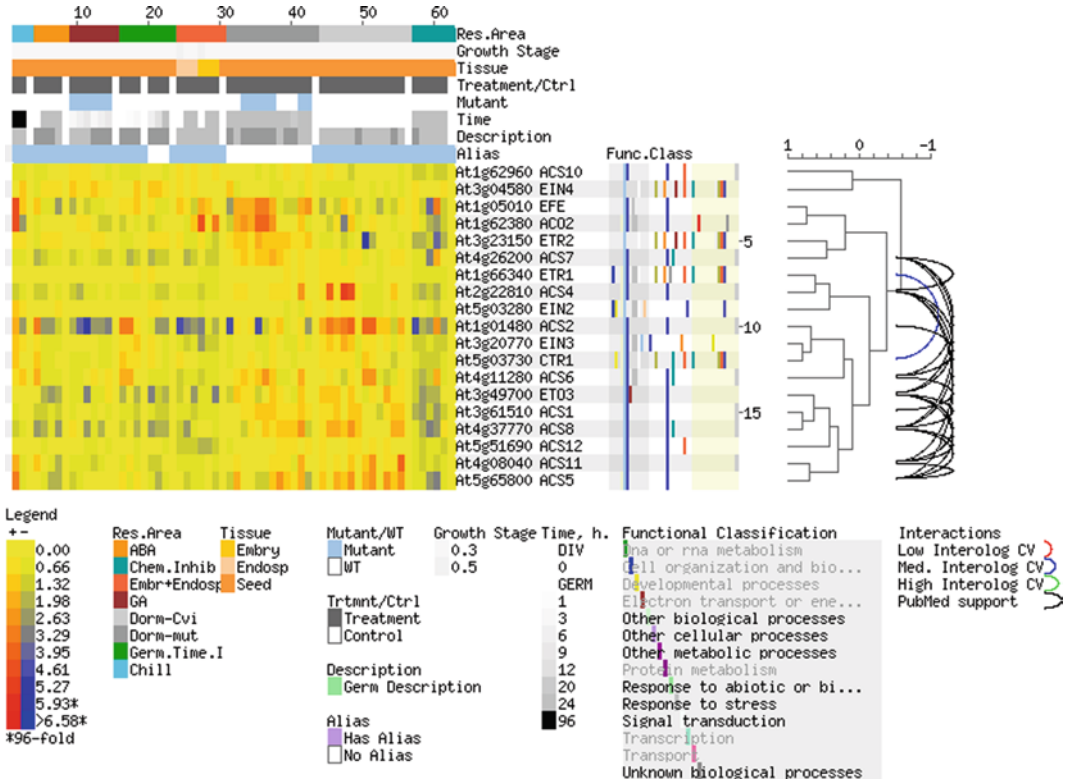

Fig. 2. Expression Browser output for a log-transformed and clustered list of genes involved in ethylene biosynthesis and response from the TAGGIT "Ethylene" category using the Seed data set. The clustering tree and protein interactions can be seen to the right of the heatmap. Many of these gene products have been documented to interact in the literature.

The arcing lines represent proven and putative interactions between the gene products, as indicated in the legend below the heatmap.

3.3. Co-expression Analysis: Expression Angler

Co-expression analysis can be considered a generalized version of more "classical" microarray analyses in which the responses of many genes under a treatment or from a specific tissue type are compared to their responses, or lack thereof, in a reference sample, with perhaps a half-dozen samples or treatments being examined. In such a "traditional" analysis, genes that are upregulated relative to their levels in a reference sample are examined for similar Gene Ontological (GO) or TAGGIT categories and unknown genes are ascribed a function based on a similar pattern of response to known genes, as described in the preceding section. A key difference between co-expression analysis and more classical microarray analyses, however, is that co-expression analysis examines a large number of data sets from published data sets while classical studies draw information from a relatively smaller number of data sets from the researcher's own experiment. Greater statistical power is

generated as a result of larger sample sizes (n = number of data sets in database) in co-expression analyses while only simple correlative data can be generated in a "classical" experiment (n = number of treatments). An additional benefit of co-expression analyses is the fact their cost is negligible, as publicly accessible microarray databases are being queried using Web-based tools, most of which are freely available. Thus, co-expressed genes of interest may be identified at the click of a mouse. This is described below:

1. Navigate to http://www.bar.utoronto.ca and select the "Expression Angler" link on the BAR homepage.
2. The Expression Angler program has a standard input page and a special input page for selecting specific samples and/or designing a custom bait. For some of the extra features of the Expression Angler program, and details concerning the design of a custom bait, see Notes 4 and 5, respectively.
3. Enter an AGI identifier in the first box. This identifier is used to extract the gene expression values associated with that gene in a specified compendium.
4. Either enter an r-value cutoff and select the "r-value cutoff range" option, or select the "Top 25, 50, or 100 hits" or the "Bottom 25, 50, or 100 hits". The Pearson correlation coefficient (r) is used to compute how similar gene expression patterns are between one's gene of interest and all the other gene expression patterns in the compendium in question. Selecting the default cutoff will return genes that are co-expressed, insofar as they meet the cutoff criterion of being the top 25 genes with the best r-values to the gene of interest while one of the other "Top" or "Bottom" options will return the specified number of genes, regardless of r-value. Those returned by the "Bottom" option are genes that are potentially responding in the opposite manner to one's gene of interest.
5. Select a compendium in which to search. There are currently eight data sets (or compendia) in which one can search: one large set from NASCArrays containing almost 400 samples, one set from the BAR in-house expression profiling service, and six data sets that are collections of data from various AtGenExpress expression profiling experiments, including one for Seeds (19). Alternately, one can upload one's own data set.
6. Click Submit and wait approximately 15 s for the BAR server to generate its output.
7. On the output page, there are a number of links. The first link to "View data set as text" leads to a text-based, tab-delimited file that one can download to open in a spreadsheet program.
8. The next two links "View formatted data set" and "View formatted data set after median centering and normalization" activate a visualization program called Data MetaFormatter

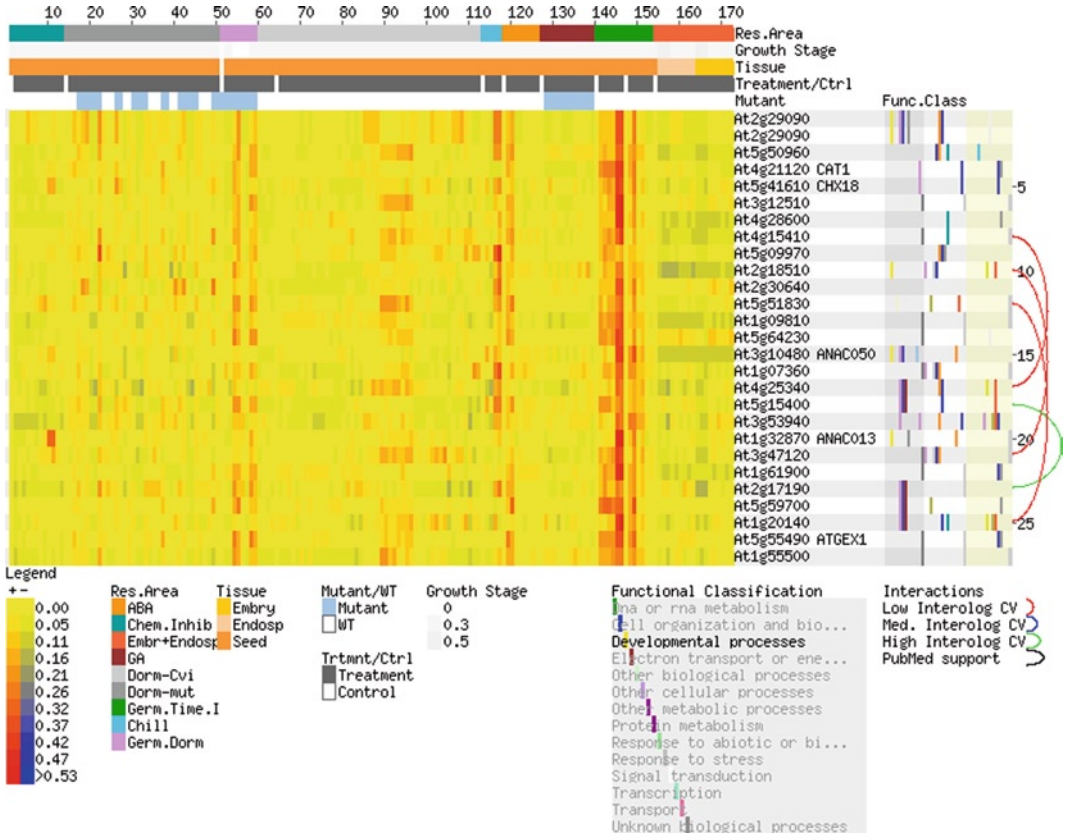

Fig. 3. An example heatmap output for a co-expression analysis using *CYP707A2*, a gene encoding an enzyme involved in ABA degradation, and the BAR's "Seed" database.

that allow one to examine graphical representations of the expression patterns of the genes passing the filtering criteria. This output is similar to that of the heatmap generated by the Expression Browser tool (Subheading 3.2). Both these outputs offer the same gene list information. In the first case, the expression values are not adjusted, while in the second case they are median-centred and normalized on a per gene basis, allowing better appreciation of the features (samples) that contribute most to the co-expression score. This can be seen in Fig. 3.

9. In descending order, genes are listed in the output according to their *r*-value score. Genes may have annotations associated with them, or be annotated as "expressed protein." By "guilt-by-association," these may also be involved in one's biological process of interest.

3.4. TAGGIT Ontology

The TAGGIT ontology was developed to provide a "biologically informed" classification of gene function for seed biology, rather than the commonly used Gene Ontology (GO) schema (20, 21).

GO terms provide information relating to Biological Process, Molecular Function and Cellular Component, but in many cases these terms are not well-defined. Therefore, an alternative ontology was developed based on prior knowledge of gene functions associated with seed maturation, dormancy, and germination. The advantage of using such a specialized ontology for the analysis of transcriptome data sets is that it can provide information about the representation of genes of known function associated with seed biology in transcriptome data sets, and also about the "state" of transcriptomes at particular developmental timepoints (by comparing the relative representation of different functional groups). Therefore, in terms of understanding transcriptome data sets, such an ontology can provide a more meaningful interpretation of data. For example, Carrera et al. (21) used this approach to show that although Arabidopsis *cts* mutant seeds do not germinate, at the transcriptome level the seeds do after-ripen, as the TAGGIT profile of stored non-germinating *cts* seeds is highly similar to that of AR wild-type seeds.

To develop the TAGGIT ontology, terms were derived from previously reported functions associated with embryo maturation, dormancy, or germination. More than 1,000 specific AGI identifiers and their associated TAIR 8 annotations were then connected to TAGGIT terms by reviewing literature associated with seed dormancy and germination – see Supplemental Data Set S1 and Supplemental Table S2 of (21). Figure 4 shows a representation of the workflow for annotating gene lists using the TAGGIT ontology terms. A comparison of terms used and a visual representation of output of the TAGGIT ontology and GO is presented in Fig. 5 for two exemplary gene sets, those upregulated in Arabidopsis L*er* dormant (D) versus after-ripened seeds. This comparison shows that analysis using TAGGIT provides a far more pronounced representation of the differences between D and AR-specific transcriptomes as compared to using the standard Gene Ontology for Arabidopsis to classify the lists. This approach has been shown to be robust using many different data sets (20–22). The TAGGIT methodology has been used both for visual representation of transcriptome states (e.g. Fig. 5), and for identifying and analyzing specific genes associated with dormancy or germination. Both approaches are described below.

3.4.1. Use of TAGGIT for Visualization of the Representation of Functional Groups in Transcriptome Data Sets

1. Download the Microsoft Excel file containing a guide to the use of TAGGIT from http://www.nottingham.ac.uk/~sbzmjh/files/TAGGITguide.xls. This file contains both the TAGGIT macro that analyses and tags gene lists and macros associated with producing graphs for visualization of tagged group representation. The TAGGIT macro itself is also available from http://www.plantphysiol.org/cgi/data/pp.107.096057/DC1/1.

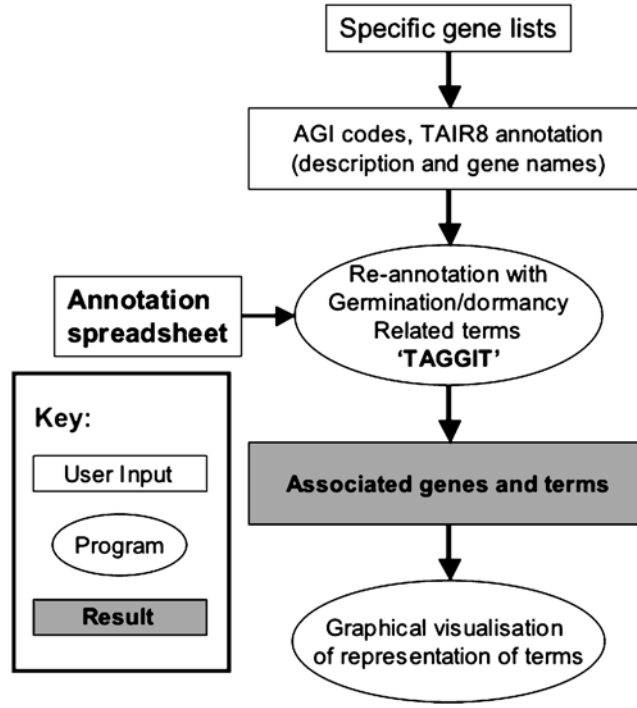

Fig. 4. Workflow associated with TAGGIT. All components are provided within the single TAGGIT guide.xls file. Specific gene lists are provided by the user.

2. In addition to macros associated with producing TAGGIT images, the Excel file contains several requisite spreadsheets:

   SUMMARY, Start Here: This spreadsheet provides a step-by-step guide to the use of TAGGIT.

   Macro: This spreadsheet is required to provide the input information as to where TAGGIT should look to find gene lists for annotation.

   TagsAndSearchTerms: This is the spreadsheet that contains all the AGI identifiers and TAIR terms used by TAGGIT to annotate gene lists.

   LerDUPvsLerAR AND LerARUPvsLerD: These are example gene lists that can be used by the operator to test that TAGGIT is functioning correctly.

3. Once familiar with the TAGGIT workflow (Fig. 4), it is possible to substitute the example gene lists for others of interest.

4. One powerful aspect of TAGGIT is the ability of the operator to substitute or change the "TagsAndSearchTerms" spreadsheet, thus providing great flexibility in what the TAGGIT macro could potentially search for. For example, it is possible to use TAGGIT to search for specific terms within a gene list (e.g. transcription factor AND light AND ethylene AND stress).

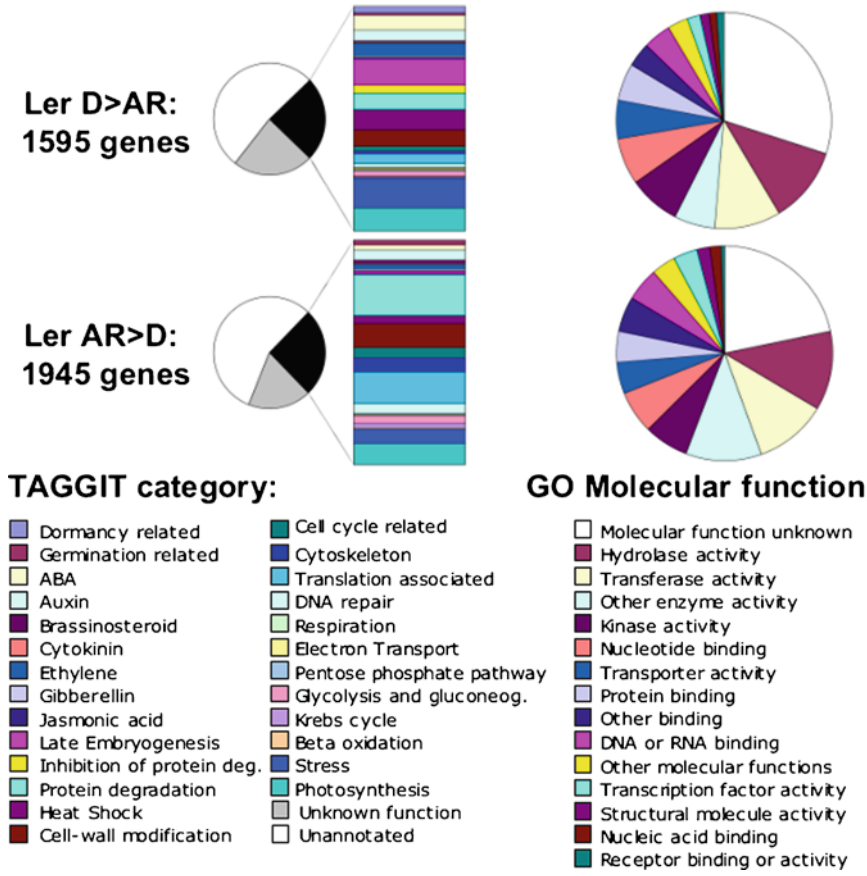

Fig. 5. Comparison of visual outputs from either the TAGGIT ontology or GO. In each case, two gene lists were analyzed. Search terms are indicated for each ontology. Ler D > AR: signifies genes more than twofold upregulated in dormant (D) compared to after-ripened (AR) Arabidopsis L*er* accession seeds at 24 h imbibition; 1,595 genes. LerAR > D: signifies genes more than twofold upregulated in AR seeds at 24 h imbibition compared to D 24-h imbibed seeds. Amounts of each category in each pie chart are proportional to the representation of each category in the gene list.

3.4.2. Integration of TAGGIT Within Bioinformatic Tools for Seed Biology

The TAGGIT ontology has been integrated into the Seed eFP Browser via a series of buttons below the eFP output representing the TAGGIT categories (19). At any point, these buttons can be clicked, producing an "Expression Browser" output consisting of the genes present in the chosen TAGGIT category within the seed gene expression data set. When a gene is queried in the eFP Browser and is present in one or more of the TAGGIT categories, the corresponding button at the bottom of the output page that represents the TAGGIT category is highlighted with a red box (Fig. 1). This allows one to know when a gene of interest has been demonstrated to function in a category covered by TAGGIT.

The use of the Seed eFP Browser is described in Subheading 3.1. Figure 1 shows an example eFP output for the gene *CYP707A2*. At the bottom of this eFP output, the TAGGIT buttons for both

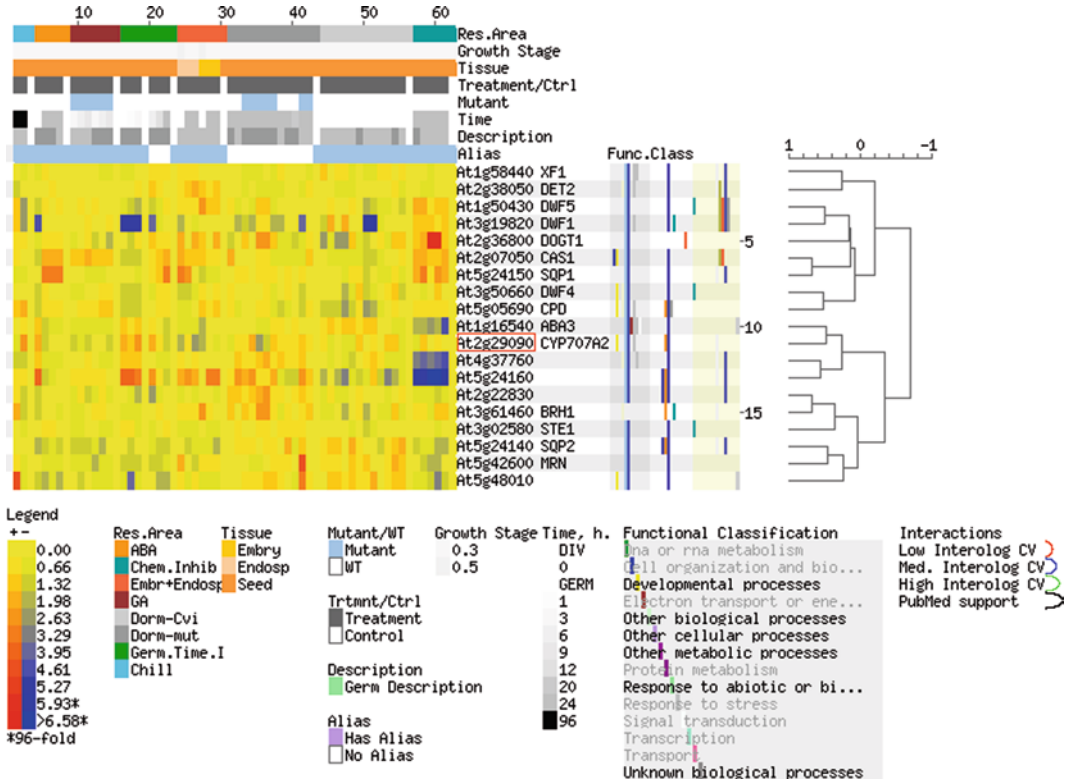

Fig. 6. Expression Browser output for the Brassinosteroid TAGGIT category following an eFP query for the gene *CYP707A2* (At2g29090). This eFP queried gene is outlined with a *red box* highlighting its position in the hierarchical clustering based on expression pattern of this TAGGIT category within the BAR's "Seed" data set.

"Abscisic Acid" and "Brassinosteroid" are highlighted with a red box, indicating the presence of this gene in both these categories. Continuing with this example, then clicking on the "Brassinosteroid" button produces the Expression Browser output for the genes within the brassinosteroid TAGGIT category, in Fig. 6. The gene that was queried in the eFP Browser, in this case *CYP707A2*, is highlighted with a red box within the gene ID list to the right of the heatmap. This indicates the presence of the gene of interest within the heatmap, whose rows have been hierarchically clustered based on the expression patterns of the genes in the TAGGIT category across the seed data sets.

3.5. Other Seed Bioinformatic Resources

Gene expression data sets examining seed germination in species other than *Arabidopsis thaliana* have been generated and deposited within public gene expression databases, such as GEO and ArrayExpress. Currently, microarray data describing rice (23), barley (24), and tomato seed germination have been made available, as shown in Table 1.

Table 1
URLs and experiment identification numbers for microarray data sets exploring rice, barley, and tomato seed germination

Organism	Gene expression database name	Database URL	Experiment ID	Platform
Rice	ArrayExpress	http://www.ebi.ac.uk/microarray-as/ae/	E-MEXP-1766	Affymetrix GeneChip Rice Genome Array
Barley	GEO	http://www.ncbi.nlm.nih.gov/geo/	GSE9365	Affymetrix GeneChip Barley Genome Array
Tomato	GEO	http://www.ncbi.nlm.nih.gov/geo/	GSE8246	TIGR Potato cDNA Array 10K

To obtain these data sets from their repositories, the following steps should be followed:

1. Point a Web browser to the appropriate URL listed in Table 1.
2. Enter the Experiment ID in the "GEO Accession" textbox for the barley and tomato data (GEO site) or the "Experiments" textbox for the rice data (ArrayExpress site).
3. Click GO or QUERY, respectively.
4. Microarray data from GEO can be downloaded using links under the "Download family" toward the bottom of the page. Data from ArrayExpress can be downloaded by clicking the "Processed" (text table) or "Raw" (.CEL) links to the right of the search result.

Both GEO and ArrayExpress provide processed gene expression data in a tab-delimited format. These processed and formatted data are ready to be imported and used in a variety of bioinformatics applications, such as the freely available Bioconductor platform (25). These tools can be used to query the data for differential expression, and in turn generate heatmaps describing the expression of groups of genes across multiple treatments. The data may also be imported in a spreadsheet program, such as Microsoft Excel, and differential expression between treatments determined using the Significance Analysis of Microarrays plugin (26). While a detailed guide to the use of these programs is beyond the scope of the chapter, it is often informative simply to be able to examine the expression pattern of an orthologous gene in another dicot species or in the monocots using a spreadsheet program.

4. Notes

1. There are several other modes of the eFP Browser beyond looking at the absolute values of a gene across different samples. It is possible to examine the "Relative" values of the gene by changing the "Mode" pulldown menu from Absolute to Relative. In the relative mode, the expression level in each sample is displayed as the ratio of its expression level relative to the median expression level across all samples, or relative to its expression value in the appropriate control sample. These ratios (fold-change above or below median or relative to the control sample) are then $\log_2$ transformed to facilitate the visualization of differences in relative signal intensity between samples. A second possibility is to change the "Mode" pulldown to "Compare", and to enter a second AGI identifier in the "Secondary AGI ID" field at the top of the screen. The eFP Browser will then display the comparative $\log_2$ transformed values of the two genes in the eFP output format, relative to their respective median or control values.
2. Some caution is advised in interpreting clustered expression data from the Expression Browser, in that some genes are more highly expressed than others. If the third output option "Average of replicate treatments relative to average of appropriate control" has been selected, the clustering analysis is performed based on the ratiometric fold-change across the different treatments. Currently, however, there is no filtering for genes with a low level of expression, including those below the suggested background level of 20 intensity units. Thus, signal intensities for different genes in the AGI identifier list may be examined for their raw (absolute) expression levels by placing the AGI IDs in the eFP Browser and reading the absolute expression level from the colour scale.
3. It is possible to handily activate the eFP Browser from the heatmap output of Expression Browser by pressing the "e" key on the keyboard and clicking on a given row in the heatmap output of the Expression Browser at the same time, or by simply clicking on the AGI ID itself in the heatmap. Alternately, AGI IDs into Expression Browser may be entered in the same order as in the log-transformed output. Select "Average of replicate treatments" output option, and then use the "unclustered results" link on the output page to get a heatmap that shows the absolute (raw) signal intensities in the same order as the $\log_2$ transformed clustered results.
4. Expression Angler has several extra features. The Subselect feature allows the user to exclude certain data sets for the calculation of the correlation coefficient: use the link to the "Subselect

and Custom Bait Page" on the Expression Angler input page, choose the data set in which you wish to select only certain samples for co-expression analysis, and on the following page, use the checkboxes to select these. In this way, it would be possible to exclude, for example, gene expression data sets from mutant seeds from the co-expression analysis.

5. Another useful feature of Expression Angler is the custom bait feature for identifying genes that are expressed only in certain samples – even if one does not have a candidate gene in mind that exhibits such an expression pattern. Follow the link to the "Subselect and Custom Bait Page" on the Expression Angler input page, and choose a data set in which to search. The "Check to enter a custom bait" checkbox should be ticked, and then subjectively high values are entered into input boxes to the left of each sample description listed towards the bottom of the Web page. The value that is typed into each box is not so important; rather it is more the "profile" of the vector that is being designed that is critical. Usually, setting the value for a sample to 100 and leaving all other values at 1 will generate a list of genes that are specific for the given treatment or tissue represented by that sample, if in fact these exist.
6. To save the output images created by any of the programs described here, move the mouse pointer over the desired image and right click (click and hold if you are using a Mac) and select the "Save picture as…" choice in the list that appears. These images then may be easily imported into popular word processing or presentation programs.

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